TaqMan® Drug Metabolism Genotyping Assays

Reference Guide

Applied Biosystems

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Preface

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Purpose of This Guide

The *TaqMan® Drug Metabolism Genotyping Assays Reference Manual* provides detailed information for use in identifying and, if necessary, troubleshooting unusual assay results. The manual provides:

- Background information on drug metabolism enzymes and polymorphisms in the genes for these enzymes, TaqMan® chemistry and the TaqMan® Drug Metabolism Genotyping Assays, to give you an overview of the basic design of the assays.
- Recommendations for preparing and performing successful TaqMan Drug Metabolism Genotyping Assays.
- Information for interpreting unexpected results in the allelic discrimination plots.

Audience

This guide is intended for lab managers and those responsible for analyzing the data from TaqMan Drug Metabolism Genotyping Assays.

Assumptions

This guide assumes that you understand how to operate your thermal cycler.

This guide also assumes that you have a working knowledge of the Sequence Detection Software (SDS) you are using to analyze your plates.

Text Conventions

This guide uses the following conventions:

- **Bold** text indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow symbol (▸) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File ▸ Open ▸ Spot Set**.

  Right-click the sample row, then select **View Filter ▸ View All Runs**.

User Attention Words

Two user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: – Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! – Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Examples of the user attention words appear below:

Note: The Calibrate function is also available in the Control Console.

IMPORTANT! To verify your client connection to the database, you need a valid user ID and password.

Safety Alert Words

Safety alert words also appear in user documentation. For more information, see "Safety Alert Words" on page x.

How to Obtain More Information

Related Documentation

The following is shipped with the assays, in portable document format (PDF):

- *TaqMan® Drug Metabolism Genotyping Assay Protocol* (PN 4362038) – Describes the protocol for performing TaqMan Drug Metabolism Genotyping Assays.

Note: To open the *TaqMan Drug Metabolism Genotyping Assay Protocol* documentation included on the CD, use the Adobe® Reader® software available from **www.adobe.com**.

The following documents are available from the Applied Biosystems web site:

- *Drug Metabolism Genotyping Assay Index,* a complete list of TaqMan Drug Metabolism Genotyping Assays
- *Ordering TaqMan® Drug Metabolism Genotyping Assays* (PN 4374203)

See the TaqMan Drug Metabolism Genotyping Assays web page at **dme.appliedbiosystems.com** for the latest information.

SDS Documentation

- *Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database User Guide 7900* (PN 4351684)
- *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide* (PN 4347822)

Thermal Cycler Maintenance and Calibration Documentation

- *7900HT Fast System Maintenance and Troubleshooting Guide* (PN 4365542)
- *Installation and Maintenance Guide for the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System* (PN 4347828).
- *GeneAmp® PCR System 9700 User's Manual, 0.5 mL Sample Block Module* (PN 04307808)
- *GeneAmp® PCR System 9700 User's Manual: Auto-Lid Dual 384 Sample Block Module* (PN 4310838)
- *Thermal Cycler Temperature Verification System: For GeneAmp® PCR System 9700: Dual 384-Well Block* (PN 04314313)
- *Thermal Cycler Temperature Verification System: For GeneAmp®PCR System 9700: 0.5 mL Block* (PN 04314777)

- *GeneAmp® PCR System 9700: 96-Well Sample Block Module User's Manual* (PN 4316011)
- *Applied Biosystems 9800 Fast Thermal Cycler: With 96-Well Aluminum Sample Block Module User Guide* (PN 4350087)
- *Thermal Cycler Temperature Verification System: For Applied Biosystems 9800 Fast Thermal Cycler User Guide* (PN 4351635)

Note: For additional documentation, see "How to Obtain Support" on page vii.

Obtaining Information from the Help System

The Sequence Detection Software has a Help system that describes how to use each feature of the user interface. Access the Help system by doing one of the following:

For the 7900 SDS:

- Click in the toolbar of the SDS Software window

  or

- Select **Help ▸ SDS Online Help**

For the 7500 SDS:

- Click in the toolbar of the SDS window

  or

- Select **Help ▸ Contents and Index**

You can use the Help system to find topics of interest by:

- Reviewing the table of contents
- Searching for a specific topic
- Searching an alphabetized index

Send Us Your Comments

Applied Biosystems welcomes your comments and suggestions for improving its user documents. You can e-mail your comments to:

techpubs@appliedbiosystems.com

IMPORTANT! The e-mail address above is only for submitting comments and suggestions relating to documentation. To order documents, download PDF files, or for help with a technical question, go to **http://www.appliedbiosystems.com**, then click the link for **Support**. (See "How to Obtain Support" below).

How to Obtain Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training

- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Safety and EMC Compliance Information

This section covers:

Safety Conventions Used in This Document x
Chemical Safety xi
Chemical Waste Safety xi
Biological Hazard Safety xi

Safety Conventions Used in This Document

Safety Alert Words

Four safety alert words appear in Applied Biosystems user documentation at points in the document where you need to be aware of relevant hazards. Each alert word—**IMPORTANT, CAUTION, WARNING, DANGER**—implies a particular level of observation or action, as defined below.

Definitions

IMPORTANT! – Indicates information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

– Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

– Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

– Indicates an imminently hazardous situation that, if not avoided, will result in death or serious injury. This signal word is to be limited to the most extreme situations.

Except for IMPORTANTs, each safety alert word in an Applied Biosystems document appears with an open triangle figure that contains a hazard symbol. *These hazard symbols are identical to the hazard symbols that are affixed to Applied Biosystems instruments*.

Examples

The following examples show the use of safety alert words:

IMPORTANT! You must create a separate sample entry spreadsheet for each 96-well plate.

The lamp is extremely hot. Do not touch the lamp until it has cooled to room temperature.

CHEMICAL HAZARD. Formamide. Exposure causes eye, skin, and respiratory tract irritation. It is a possible developmental and birth defect hazard. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

ELECTRICAL HAZARD. Failure to ground the instrument properly can lead to an electrical shock. Ground the instrument according to the provided instructions.

For Additional Information

Please see the Saftey chapters in:

- The *TaqMan® Drug Metabolism Genotyping Assay Protocol* (PN 4362038)
- The user guides for the thermal cycler and SDS software you use to perform the assays.

Chemical Safety

Chemical Hazard Warning

CHEMICAL HAZARD. Before handling any chemicals, refer to the Material Safety Data Sheet (MSDS) provided by the manufacturer, and observe all relevant precautions.

About MSDSs

Chemical manufacturers supply current Material Safety Data Sheets (MSDSs) with shipments of hazardous chemicals to *new* customers. They also provide MSDSs with the first shipment of a hazardous chemical to a customer after an MSDS has been updated. MSDSs provide the safety information you need to store, handle, transport, and dispose of the chemicals safely.

Each time you receive a new MSDS packaged with a hazardous chemical, be sure to replace the appropriate MSDS in your files.

Chemical Waste Safety

Chemical Waste Hazard

HAZARDOUS WASTE. Refer to Material Safety Data Sheets and local regulations for handling and disposal.

Biological Hazard Safety

BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Follow all applicable local, state/provincial, and/or national regulations. Wear appropriate protective equipment, which includes but is not limited to: protective eyewear, face shield, clothing/lab coat, and gloves. All work should be conducted in properly equipped facilities using the appropriate safety equipment (for example, physical containment devices). Individuals should be trained according to applicable regulatory and company/institution requirements before working with potentially infectious materials. Read and follow the applicable guidelines and/or regulatory requirements in the following:

- U.S. Department of Health and Human Services guidelines published in *Biosafety in Microbiological and Biomedical Laboratories* (stock no. 017-040-00547-4; **http://bmbl.od.nih.gov**)
- Occupational Safety and Health Standards, Bloodborne Pathogens (29 CFR§1910.1030; **http://www.access.gpo.gov/ nara/cfr/waisidx_01/ 29cfr1910a_01.html**).
- Your company's/institution's Biosafety Program protocols for working with/handling potentially infectious materials.

Additional information about biohazard guidelines is available at:

http://www.cdc.gov

Part I

Background Information

1

Introduction

An understanding of assay design will help you identify unusual results and troubleshoot problems you encounter when running TaqMan® Drug Metabolism Genotyping Assays.

This chapter covers:

Introduction to the Drug Metabolism Enzyme Genes 1-2
Assay Development and Testing 1-3
Overview of TaqMan® Probe-Based Chemistry 1-8

Introduction to the Drug Metabolism Enzyme Genes

Drug metabolizing enzymes (DMEs) are proteins involved in the biotransformation, metabolism, and/or detoxification of endogenous and foreign compounds (for example, naturally occurring compounds like prostaglandins, drugs and environmental agents). Genes that code for these enzymes are often referred to as "DME genes." Polymorphisms in the DME genes may influence the rate of foreign compound metabolism and/or excretion among individuals, thereby potentially affecting foreign compound efficacy and/or toxicity. Several of these polymorphisms may affect drug efficacy and toxicity (Eichelbaum, 2006 and Roses, 2004).

For example, there are 78 known variants of the CYP2D6 gene. Some of these variations result in a complete loss of enzymatic activity while some of the variants only reduce catalytic activity (Xie H-G, 2002).

TaqMan® Drug Metabolism Genotyping Assays are designed to detect polymorphisms such as SNPs, insertions and deletions (indels), and multi-nucleotide polymorphisms (MNPs) in the genes that code for the drug metabolism enzymes and drug transporters. The terms "SNP" and "polymorphism" will be used interchangeably throughout the rest of this document and refer to all of these polymorphisms.

Drug Metabolism Enzyme Classifications

The products of DME genes are Phase I and Phase II drug metabolism enzymes, transmembrane transporters, and other gene products thought to be involved in the metabolism of endogenous compounds and xenobiotics in humans.

- **Phase I enzymes** – Involve the hydrolysis, oxidation, or reduction of xenobiotic compounds. These enzymes either create or expose a functional group (such as a hydroxyl group or carboxyl group) on the target small molecule. Examples of Phase I enzymes are the cytochrome P450 gene super-family and the alcohol dehydrogenase gene family. Four members of the P450 super-family, CYP3A, CYP2D6, CYP2C19, and CYP2C9, account for almost 50% of metabolism of commonly used drugs (Wilkinson, 2005).
- **Phase II enzymes** – Involve the conjugation of various cofactors to functional groups on small molecules that are often exposed or created by a Phase I reaction. Phase II reactions include glucuronidation, acetylation, and sulfation. Phase II reactions often increase the hydrophilicity of a compound, thereby enhancing its excretion from the body. This class includes the UDP-glucuronosyl transferase (UGT) family that metabolizes morphine and thiopurine S-methyltransferase (TMPT) that metabolizes Captropril (Shastry, 2006).
- **Drug transporters** – Expressed in liver, kidney, and the intestines, involved in the elimination of xenobiotics from the body. This class of protein includes organic anion transporters, organic cation transporters, peptide transporters, and nucleoside transporters. Many of these proteins exhibit broad substrate specificity which can lead to significant drug-drug interactions at the transporter level. Examples of transporters are: the multidrug resistance-associated protein MRP1 (ABCC1), ATP-dependent transporters like MDR1 (ABCB1; P-glycoprotein), organic anion transporters such as hOAT1 (SLC22A6) (Bleasby 2005), and multidrug resistance protein MRP2 (ABCC2) (Szakács, 2004).

Allele Nomenclature

Allele nomenclature for the polymorphisms in the DME genes follows an international system created to describe polymorphisms in DNA and protein sequences (den Dunnen, 2001). When available, Applied Biosystems provides the allele nomenclature for the SNP being interrogated by a particular TaqMan Drug Metabolism Genotyping Assay. The allele nomenclature indicates the gene name, the allele designation, and the base changes associated with the polymorphism. Because this nomenclature is used in the Applied Biosystems web site for categorizing the TaqMan Drug Metabolism Genotyping Assays, you can search for an assay using the allele nomenclature for the SNP of interest.

An example of this nomenclature is CYP1A1*1C, g.–3229G>A, which describes a polymorphism in a cytochrome P450 gene, where:

- "CYP1A1" indicates the HUGO gene name
- "*1C" indicates the allele from the Human Cytochrome P450 Nomenclature Committee (web page listed below)
- "g" indicates that the mapping position comes from the genomic DNA reference sequence ("c" indicates cDNA)
- "–3229" indicates the nucleotide position relative to the first base of the start codon on the genomic DNA reference sequence
- "G>A" indicates the nucleotide or base change

Note: The order of the alleles does not reflect frequency and/or a wild-type versus mutation designation, because they can differ between populations.

The allele nomenclature can indicate *one* SNP or *multiple* SNPs associated with a haplotype. The TaqMan Drug Metabolism Genotyping Assays are designed to identify a single polymorphism within an "allele," which is generally a haplotype. To investigate an allele from the common allele nomenclature sites, you may need several TaqMan Drug Metabolism Genotyping Assays (one for each SNP in the haplotype).

Allele nomenclature was gathered from the following sites:

- The human cytochrome P450 (CYP) allele nomenclature committee web site: **www.imm.ki.se/cypalleles/**
- The arylamine *N*-acetyltransferase *(NAT)* nomenclature web site: **www.louisville.edu/medschool/pharmacology/NAT.html**
- The home page of the committee mediating the naming of UDP glucuronosyltransferase: **som.flinders.edu.au/FUSA/ClinPharm/UGT/**

For general information on polymorphism nomenclature, go to: **www.gene.ucl.ac.uk/nomenclature/guidelines.html**

Note: Because not all the genes or polymorphisms included in TaqMan Drug Metabolism Genotyping Assays have been curated on a nomenclature site, not all of them are associated with public allele nomenclature.

Assay Development and Testing

This section provides general information about how Applied Biosystems designed and tested the TaqMan Drug Metabolism Genotyping Assays.

Bioinformatics Evaluation and Design

Applied Biosystems performed extensive bioinformatics to ensure that all SNPs in the TaqMan Drug Metabolism Genotyping Assays were properly mapped to the human genome assembly. The most current mapping information is available on the TaqMan Drug Metabolism Genotyping Assays page on the Applied Biosystems web site: **dme.appliedbiosystems.com**.

1. Approximately 220 genes were selected based on their roles in drug metabolism and transport. A complete list of genes can be found in the TaqMan Drug Metabolism Genotyping Assay Index, available on the Applied Biosystems web site.
2. Polymorphisms associated with DME genes were identified from multiple sources, including dbSNP, HapMap, collaborators, public allele nomenclature sites and Applied Biosystems proprietary database.
3. For each potential assay, the polymorphism and 300 bases on either side of it were mapped to the human genome. A polymorphism was considered to be successfully mapped when it was aligned to only one location in the genome.
4. Polymorphisms were selected for inclusion into the TaqMan Drug Metabolism Genotyping Assays collection based on their location on the reference genome. SNPs in coding regions (mis-sense polymorphisms, non-sense polymorphisms, silent polymorphisms) as well as SNPs in splice site acceptor regions, splice site donor regions, and the 5' UTR were chosen. Additional intronic polymorphisms were included only when these were available from the public allele nomenclature web sites.
5. For all polymorphisms, repetitive regions, nontarget polymorphisms, and putative polymorphisms within 300 bases on either side of the SNP of interest were masked to avoid assays with potential polymorphisms under probes and primers.
6. The masked sequence was sent to the Applied Biosystems proprietary TaqMan Assay Design Software.
7. The resulting assay design was mapped to the genome again, using the BLAST database search algorithm, to ensure the primers and probes are specific for the target polymorphism.
8. If the assay design mapped with sufficient specificity, it was synthesized and submitted for wet lab testing (see "Wet Testing of Assays" on page 1-6). Assays that failed wet lab testing were resubmitted to the assay design software to generate alternative assay designs.

Development of the Thermal Cycler Method

To address the homology issues within the DME genes and to ensure specificity of these assays, Applied Biosystems created assay designs that resulted in amplicon lengths greater, in general, than those for other TaqMan Genomic Assay product lines. The average amplicon length for the TaqMan Drug Metabolism Genotyping Assays is 160 base pairs, compared to 100 base pairs for other Applied Biosystems TaqMan genotyping products. Wet testing of these longer amplicons showed that the Rn values (normalized signal) were often lower for these longer amplicons, affecting cluster separation. To address this issue, Applied Biosystems developed a new thermal cycler method. The development of this new thermal cycler method is described below.

Determination of the Optimal Number of Cycles

A representative group of assays were subdivided by amplicon length into three groups: short (<100 bases), medium (100-110 bases) and long (>110 bases). The normalized signals for each set of assays, performed at 40 and 50 cycles with a 60 second extension, were compared. At 50 cycles, the average signal for both the VIC® and FAM™ dyes increased dramatically for the long amplicons. For these assays, 50 cycles produced better genotyping data than the traditional 40 cycles.

Determination of the Optimal Extension Time

To further improve assay performance, Applied Biosystems investigated longer extension times. Three different extension times (60, 90 and 120 seconds) for 50 cycles were investigated. The data produced by these experiments indicated that the 90 second extension showed significant increases in the normalized signal; the average signal increased for both the VIC and FAM dyes. No significant increases were observed with the 120 second extension.

Clustering was also analyzed, and the group of assays with the longest amplicons showed the most improvement in tightness and angle separation of the genotype clusters. Tightness and angle separation are determined algorithmically; tightness is the distance of individual points from each other and angle separation measures the distance between clusters.

Figure 1-1 demonstrates the effects of increased number of cycles and longer extension time on the assays based on amplicon length.

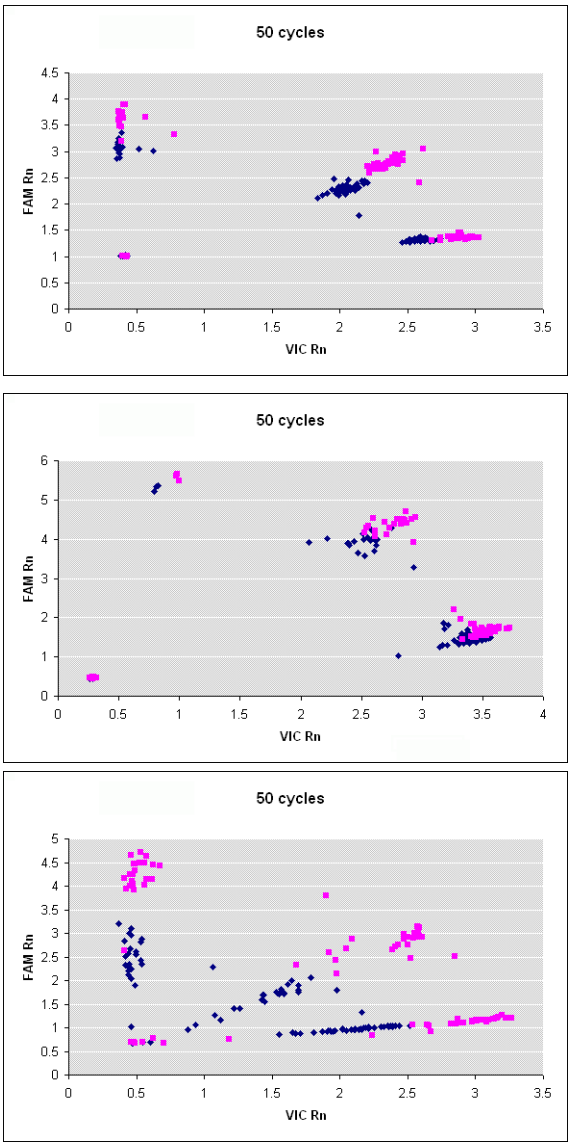


Figure 1-1 Allelic discrimination plots for short, medium, and long amplicon assays for 60- (dark blue) and 90-second (pink) extension times

Wet Testing of Assays

Stock DNA samples were obtained from Coriell Cell Repositories (CCR, **www.coriell.org/ccr/ccrsumm.html**). Dilutions of the stock DNA were quantitated by the RNase P method. Each stock DNA was then diluted with water to yield a 1 ng/µL working stock. 3 µL (3 ng total DNA) of each DNA was delivered to the wells of a 384-well microtiter plate. Each plate was dried overnight and the plates were sealed and stored for up to a year prior to use.

Reaction mixes were prepared for 5 µL reactions. PCR was performed using GeneAmp® PCR System 9700, as described in the *TaqMan® Drug Metabolism Genotyping Assays Protocol* (PN 4362038 Rev. A). Endpoint data were collected using the Applied Biosystems 7900HT Real-Time PCR System.

All assays were wet-tested on duplicate plates, containing African American and Caucasian DNA samples. Each plate contained DNA samples from:

- 45 African Americans
- 46 Caucasians
- 3 No template controls (water)

Each assay was run twice to confirm performance. Parameters used to analyze the assays included: cluster signal, tightness, angle separation, and Hardy-Weinberg equilibrium.

If good performance was observed, assays were run on a proprietary set of DNA samples from Chinese and Japanese populations to generate allele frequencies for those populations. (Each set containing 45 samples was provided as part of a collaboration. These sets are not publicly available.)

If an assay performed poorly, it was submitted to the assay design software for redesign. Due to limitations in the technology and/or the type of polymorphism, an assay could not be developed for every polymorphism in the drug metabolism enzyme genes.

Other Assays for the DME Genes

Approximately 220 genes were included in TaqMan Drug Metabolism Genotyping Assay set. Genes that are not part of the TaqMan Drug Metabolism Genotyping Assay collection may be found in the Applied Biosystems TaqMan® SNP Genotyping Assays product line. The TaqMan Drug Metabolism Genotyping Assays include polymorphisms found in coding regions, splice junctions, or regulatory elements for the DME genes. Additional intronic SNPs were included only if evidence from the public allele nomenclature web sites was identified.

If your SNP of interest is not available as a TaqMan Drug Metabolism Genotyping Assay, it may be available as a Applied Biosystems TaqMan SNP Genotyping Assay. This pre-designed assay collection includes over four million assays. Search for the gene of interest at the web site **snp.appliedbiosystems.com**.

Overview of TaqMan® Probe-Based Chemistry

This section explains how the TaqMan Drug Metabolism Genotyping Assays work during PCR.

Assay Components

Each TaqMan Drug Metabolism Genotyping Assay consists of a single, ready-to-use tube containing:

- Two sequence-specific primers for amplifying the polymorphism of interest
- Two allele-specific TaqMan® MGB probes for detecting the alleles for the specific polymorphism of interest

The tube contains enough reagent to perform 750 assays at a 5 µL reaction volume (384-well plate) or 150 assays at a 25 µL reaction volume (96-well plate). All TaqMan Drug Metabolism Genotyping Assays are run using the same instrument settings.

About the Probes

Each allele-specific TaqMan MGB probe has:

- A reporter dye at its 5′ end
  - VIC® dye is linked to the 5′ end of the Allele 1 probe.
  - FAM™ dye is linked to the 5′ end of the Allele 2 probe.

    The Allele 1 VIC dye-labeled probe corresponds to the first nucleotide inside the square brackets of the context sequence in the Assay Information File (AIF) on the CD-ROM shipped with each assay order. The Allele 2 FAM dye-labeled probe corresponds to the second nucleotide inside the square brackets of the context sequence in the AIF. For the context sequence ATCGATT[G/T]ATCC, the VIC dye-labeled probe binds to the allele containing G and the FAM dye-labeled probe binds to the allele containing T.
- A minor groove binder (MGB), which increases the melting temperature (T_m) for a given probe length, allows the design of shorter probes (Alfonina et al., 1997, Kutyavin et al., 1997). The presence of the MGB results in greater differences in T_m values between matched and mismatched probes. These differences produce more robust allelic discrimination.
- A nonfluorescent quencher (NFQ) at its 3′ end which allows detection of reporter dye contributions with greater sensitivity.

5′ Nuclease Assay

Figure 1-2 is a schematic depiction of the 5′ nuclease assay. During PCR:

- Each TaqMan MGB probe anneals specifically to its complementary sequence between the forward and reverse primer sites.
- When the oligonucleotide probe is intact, the proximity of the quencher dye to the reporter dye causes the reporter dye signal to be quenched.
- AmpliTaq Gold® DNA polymerase extends the primers bound to the genomic DNA template.
- AmpliTaq Gold DNA polymerase (a 5′ nuclease) cleaves probes that are hybridized to the target sequence.
- When the hybridized probes are cleaved by AmpliTaq Gold DNA polymerase, the quencher dye is separated from the reporter dye, increasing the fluorescence of the reporter dye. Therefore, the fluorescence signal generated by PCR amplification indicates which alleles are present in the sample.

Minimizing Non-Specific Fluorescence

In TaqMan assays, fluorescence from nonspecifically bound probes is reduced because nucleotide mismatches between a probe and a sequence reduce the chances that the probe will be cleaved. The probe's short length means a one base-pair mismatch has a larger negative effect on the binding than for a longer probe. The mismatched probe does not bind tightly to the allele, allowing the AmpliTaq Gold® DNA polymerase to displace the probe without cleaving the dye.

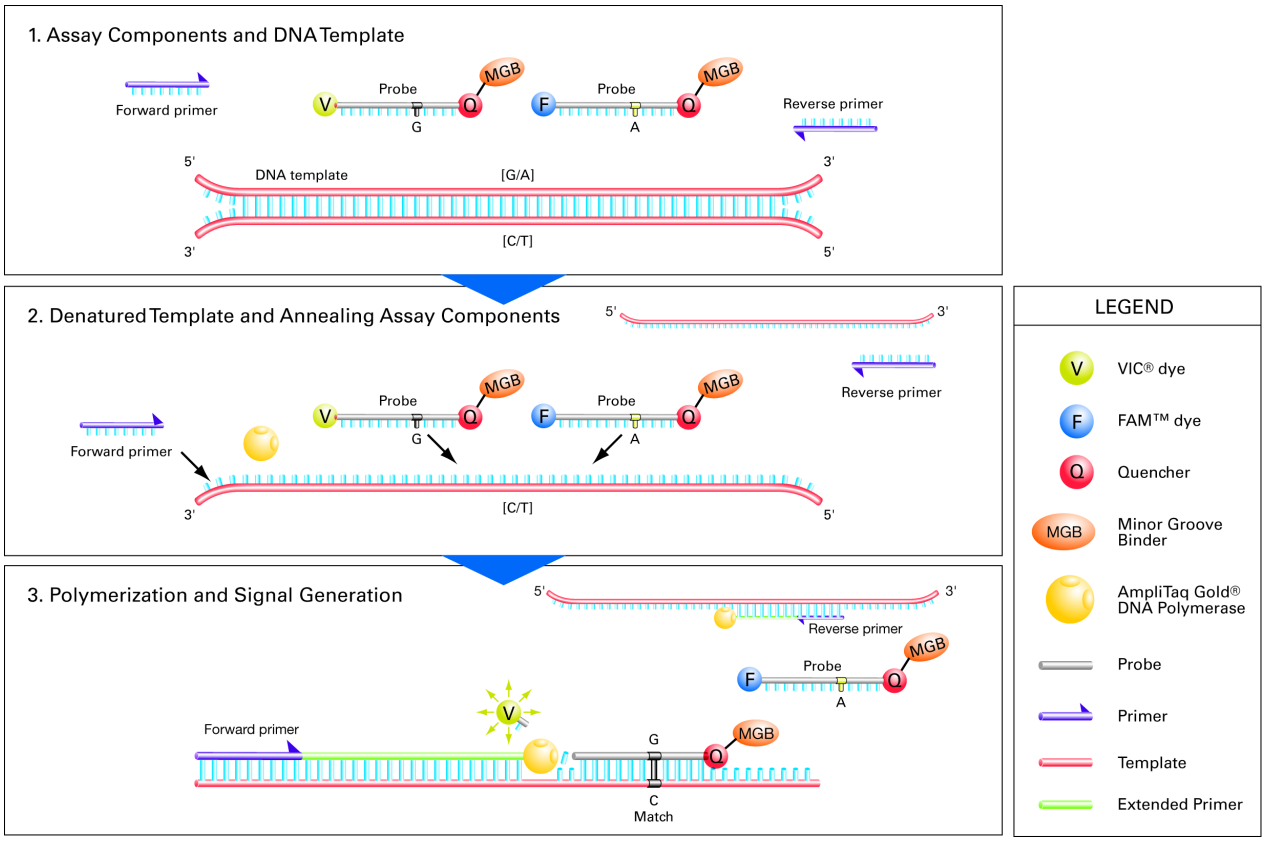


Figure 1-2 Schematic depiction of a TaqMan® Drug Metabolism Genotyping Assay

Reading the Plates

TaqMan Drug Metabolism Genotyping Assays are read at the PCR endpoint. DNA samples on 96- or 384-well plates are genotyped simultaneously. Genotype calls for individual samples are made by plotting the normalized intensity of the reporter dyes in each sample well on an allelic discrimination plot (Figure 1-3 on page 1-10). An algorithm in the data analysis software assigns individual sample data to a particular cluster and makes the genotype calls.

Note: The clustering algorithm used in the data analysis software does not call genotypes when only one cluster is present.

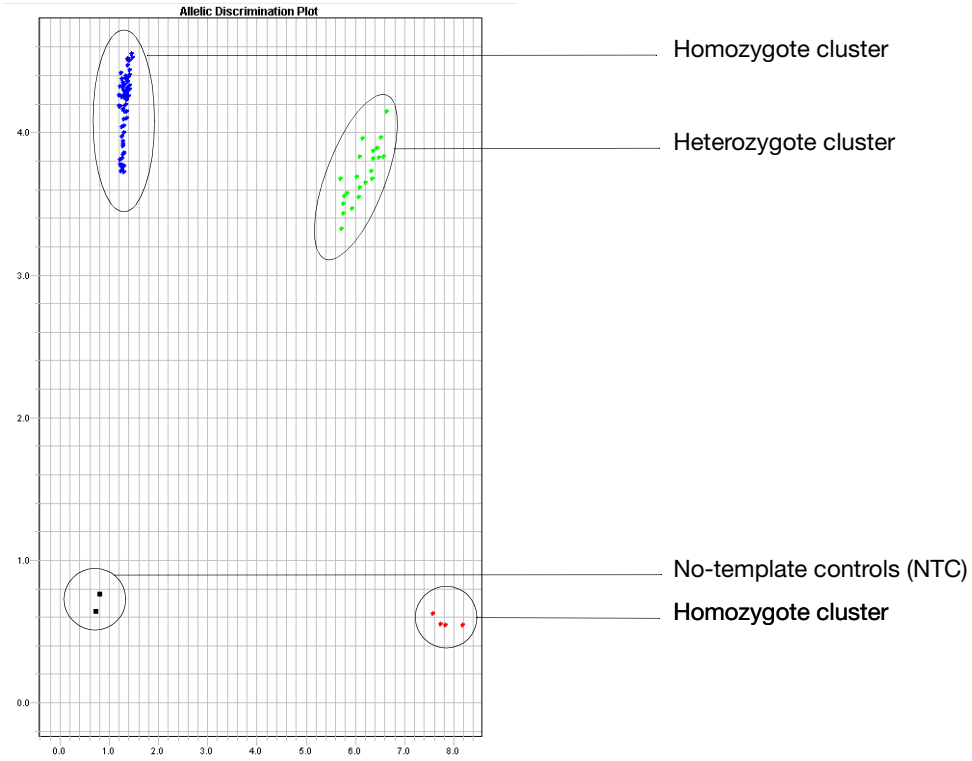


Figure 1-3 Example of an allelic discrimination plot for a TaqMan® Drug Metabolism Genotyping Assay

The table below shows the correlation between fluorescence signals and sequences in a sample.

A substantial increase in…	Indicates…
VIC® dye fluorescence only	Homozygosity for Allele 1
FAM™ dye fluorescence only	Homozygosity for Allele 2
Both VIC and FAM fluorescence	Allele 1-Allele 2 heterozygosity

2

Best Practices for Running Assays

This chapter provides you with recommendations for successfully setting up and running the assays. Follow these recommendations and the instructions in the *TaqMan® Drug Metabolism Genotyping Assay Protocol*, to lessen the need for post-assay troubleshooting.

This chapter covers:

Assay Conditions 2-2
Recommended Thermal Cyclers 2-5
Introduction to Markers, Detectors and Tasks 2-5
Detectors, Markers and Tasks for the 7900HT Fast Real-Time PCR System 2-6
Detectors, Markers and Tasks for 7300/7500/7500 Fast Real-Time PCR Systems . . . 2-10

Assay Conditions

IMPORTANT! Conditions for the TaqMan® Drug Metabolism Genotyping Assays differ from the conditions for other TaqMan® SNP Genotyping Assays, particularly the thermal cycler conditions. Refer to the sections below and the *TaqMan® Drug Metabolism Genotyping Assay Protocol* (PN 4362038) for more details.

Note: You can also use the TaqMan Drug Metabolism Genotyping Assays thermal cycling protocol for any TaqMan SNP Genotyping Assay, but the run time is longer. If you run TaqMan Drug Metabolism Genotyping Assays and TaqMan SNP Genotyping Assays on the same plate, you should use the TaqMan Drug Metabolism Genotyping Assays thermal cycling protocol.

PCR Good Laboratory Practices

PCR assays require special laboratory practices to avoid false positive amplifications (Kwok and Higuchi, 1989). The high throughput and repetition of these assays can lead to amplification of a single DNA molecule (Saiki et al., 1985; Mullis and Faloona, 1987).

- Wear a clean lab coat (not previously worn while handling amplified PCR products or used during sample preparation) and clean gloves when preparing samples for PCR amplification.
- Change gloves whenever you suspect that they are contaminated.
- Maintain separate areas, dedicated equipment, and supplies for:
  - Sample preparation and PCR setup
  - PCR amplification and post-PCR analysis
- Never bring amplified PCR products into the PCR setup area.
- Open and close all sample tubes and reaction plates carefully. Do not splash or spray PCR samples.
- Keep reactions and components sealed as much as possible.
- Use positive displacement pipettes or aerosol-resistant pipette tips.
- Clean lab benches and equipment periodically with freshly diluted 10% bleach solution.

DNA Preparation

Applied Biosystems recommends using 3 to 20 ng of purified genomic DNA in the TaqMan Drug Metabolism Genotyping Assays. Applied Biosystems also recommends that you use the same amount of DNA for all samples run with one assay. These assays were wet-tested with 3 ng of DNA. If you use less than 3 ng, you must verify the robustness of the assay in your own lab.

Applied Biosystems recommends two quantitation methods:

- The TaqMan® RNase P Detection Reagents (PN 4316831). You may use your own DNA samples or the TaqMan® DNA Template Reagents (PN 401970) to create a standard curve. Refer to the tutorial *Creating Standard Curves with Genomic DNA or Plasmid DNA Templates for Use in Quantitative PCR*, available from the Applied Biosystems web site.

  or

- UV absorbance (A_{260}/A_{280}) measurements.

Note: The TaqMan RNase P method is preferred because it is more accurate and it assesses sample quality.

Commercial Products for DNA Preparation

Table 2-1 lists many commercial products that you can use to prepare genomic DNA templates. This is not an exhaustive list, and you may find other products that work. Applied Biosystems makes no specific recommendations on the use of these products. Follow the manufacturer's instructions to prepare the DNA templates for PCR.

Table 2-1 Commercial products for preparing genomic DNA

Product	Source	Description
BloodPrep® DNA	Applied Biosystems	Purify genomic DNA from blood, tissue, tissue culture, or buccal swabs
Genomix 10 mL Blood	Talent	Whole blood, buffy coat, unpreserved blood
Genomix 2.4 mL Blood	Talent	Whole blood
Genomix Cells and Tissue	Talent	Tissue
PAXgene Blood DNA Kit	Qiagen	Blood collection and purification system
Puregene® DNA Purification Kit	Gentra	Blood, bone marrow, packed cells, buffy coat
QIAamp DNA Blood Mini Kit	Qiagen	Blood and cell free body fluids
QuickGene-810	FUJIFILM Tokyo	Automated system for purifying gDNA from blood or tissue
Wizard® Genomic DNA Purification Kit	Promega	Purify genomic DNA from blood, tissue, or tissue culture

You can use the ABI PRISM® 6100 Nucleic Acid PrepStation to isolate and purify genomic DNA from a variety of biological samples.

Recommended Controls

- A no-template control (NTC)
- A positive control

The NTC orients the VIC® dye and/or FAM™ dye clusters to an origin. It also allows for the detection of any genomic DNA or PCR amplicon contamination on a set of plates.

A positive control (a sample with a known genotype) helps you assess the performance of an assay. In some cases, you can purchase genomic DNA from Coriell Cell Repositories (**www.coriell.org/ccr/ccrsumm.html**) with a known genotype. Contact Applied Biosystems for the Repository ID (needed for ordering from Coriell) and genotype for the sample.

Thermal Cycler Method

The recommended thermal cycler method for assays using TaqMan® Universal PCR Master Mix without or with AmpErase® UNG is shown in Tables 2-2 and 2-3, below. Numbers in red indicate the changes from the standard conditions for other TaqMan SNP Genotyping Assays. See "Development of the Thermal Cycler Method" on page 1-4 for information on how this method was developed.

Table 2-2 Thermal cycler method for TaqMan® Universal PCR Master Mix *without* AmpErase® UNG, for TaqMan Drug Metabolism Genotyping Assays

Temperature (°C)	Time (minutes:seconds)	Cycles
95	10:00	Hold
92	00:15	50
60	01:30	

Table 2-3 Thermal cycler method for TaqMan® Universal PCR Master Mix *with* AmpErase® UNG, for TaqMan Drug Metabolism Genotyping Assays

Temperature (°C)	Time (minutes:seconds)	Cycles
50	02:00	Hold
95	10:00	Hold
92	00:15	50
60	01:30	

Recommended Thermal Cyclers

Applied Biosystems recommends using Applied Biosystems thermal cyclers and Sequence Detection Systems to perform and collect data from your assays. Applied Biosystems instruments that can be used for PCR amplification are shown below.

Instrument Type	Instrument Name
Thermal Cycler	Applied Biosystems 9800 Fast Thermal Cycler, using the 9700/9600 emulation mode **Note:** TaqMan® Drug Metabolism Genotyping Assays can be performed on a 9800 Fast Thermal Cycler using *standard* reagents and *standard* cycling methods. TaqMan Drug Metabolism Genotyping Assays are not supported using Fast reagents or Fast protocols.
	GeneAmp® PCR System 9700
Real-Time PCR System These instruments allow you to perform PCR amplification and then perform the endpoint plate read separately.	Applied Biosystems 7900HT Fast Real-Time PCR System. **Note:** TaqMan Drug Metabolism Genotyping Assays can be performed on a 7900HT Fast System using *standard* reagents and *standard* cycling methods. TaqMan Drug Metabolism Genotyping Assays are not supported using Fast reagents or Fast protocols.
	Applied Biosystems 7900HT Real-Time PCR System
	Applied Biosystems 7500 Fast Real-Time PCR System **Note:** TaqMan Drug Metabolism Genotyping Assays can be performed on a 7500 Fast System using *standard* reagents and *standard* cycling methods. TaqMan Drug Metabolism Genotyping Assays are not supported using Fast reagents or Fast protocols.
	Applied Biosystems 7500 Real-Time PCR System
	Applied Biosystems 7300 Real-Time PCR System
	ABI PRISM® 7000 Sequence Detection System

Note: Applied Biosystems does not recommend using real-time data to call genotypes.

Introduction to Markers, Detectors and Tasks

Setting up and selecting detectors, markers and tasks will directly affect the perceived success of your assay. If you do not set up the detectors, markers and tasks correctly, you run the risk of obtaining incorrect or no results in your allelic discrimination plot.

- **Detector** –A software representation of a TaqMan® probe and primer set and associated fluorescent dye that detects *a single allele*.
- **Marker** – A set of two detectors that discriminate between different alleles of a common locus. Allele 1 is detected by one detector (for example, VIC dye), and allele 2 is detected by the second detector (for example, FAM dye).

- **Tasks** – The task defines the specific purpose or function of the wells on the plate. You must assign a "task" to each well of the plate document. For genotyping there are two tasks, "unknown" and "NTC" (no template control). The SDS software uses the detector task assignments to determine how to treat the data produced by the wells when analyzing the run data.

IMPORTANT! Each assay needs its own marker. If you run more than one assay per plate, the Sequence Detection Software requires that each assay be assigned a unique marker name. Mixed markers result in undetermined calls.

Detectors, Markers and Tasks for the 7900HT Fast Real-Time PCR System

Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

For More Information

See the *SDS Enterprise Database for the Applied Biosystems 7900HT Fast Real-Time PCR System and SDS Enterprise Database and User Guide* (PN 4351684 Rev. A).

User Access Requirement

To use the SDS Enterprise Database, you must belong to the Scientist or Administrator User Group to create and apply detectors and markers.

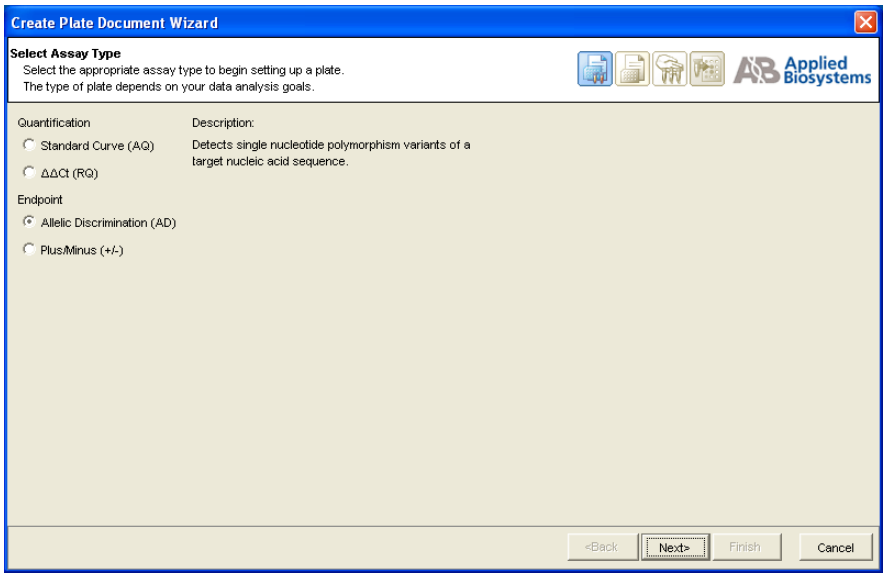
Creating Detectors

Before you can use a plate document to run a plate, it must be configured with detector and marker information. Use the wizard and import the relevant information from the AIF shipped on a CD with your TaqMan Drug Metabolism Genotyping Assay.

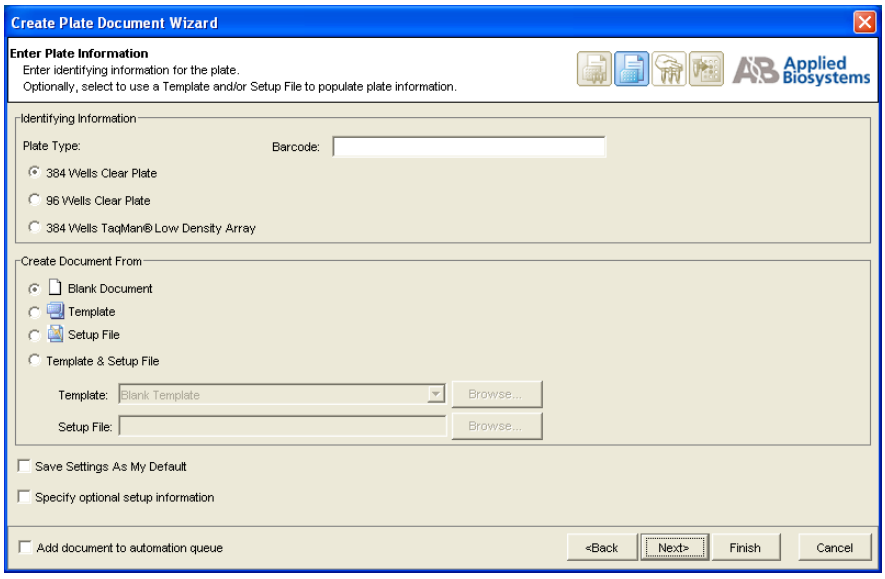
IMPORTANT! Locate the CD with the AIF and insert it into the CD drive before performing this procedure.

1. Select **New** ▸ Plate Document.

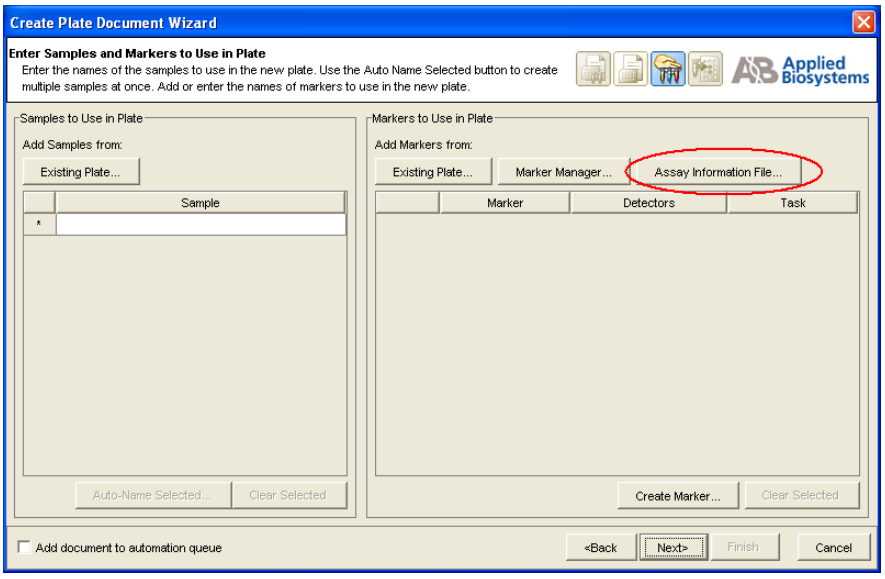
2. For the assay type, click **Allelic Discrimination** and then **Next.**



3. Click the number of wells for your plate, choose **Blank Document** and then click **Next.**

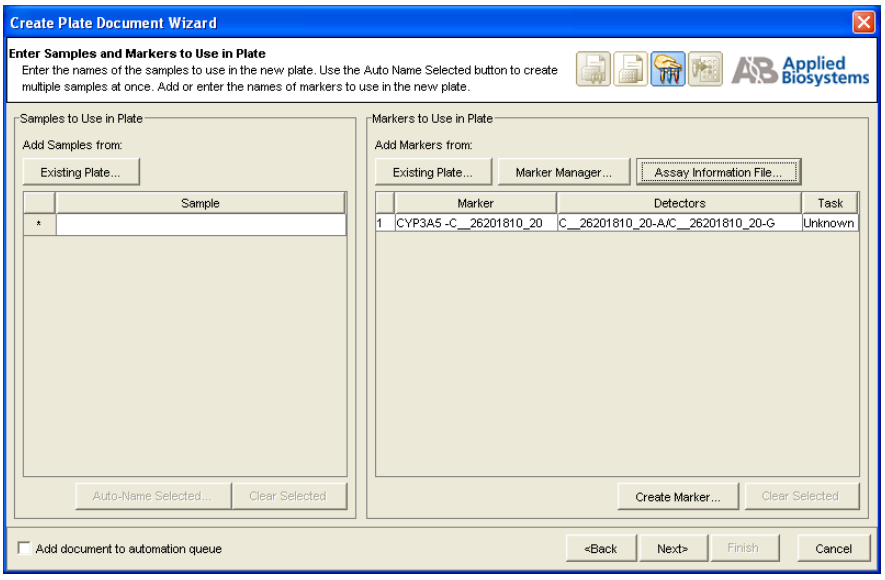


4. In the Enter Samples and Markers to Use in Plate page, click **Assay Information File**.

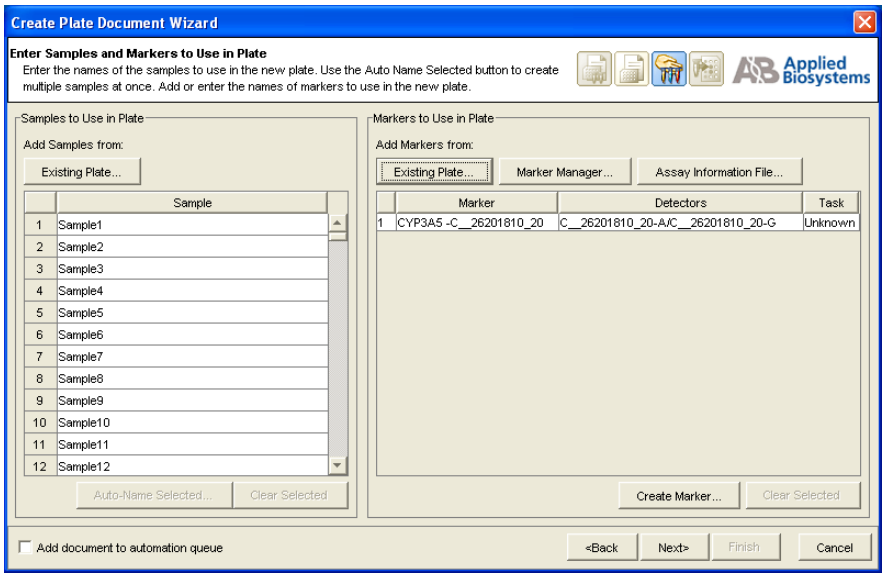

Create Plate Document Wizard
Enter Samples and Markers to Use in Plate
Enter the names of the samples to use in the new plate. Use the Auto Name Selected button to create multiple samples at once. Add or enter the names of markers to use in the new plate.
Applied Biosystems
Samples to Use in Plate
Add Samples from:
Existing Plate...
Sample
Auto-Name Selected...
Clear Selected
Markers to Use in Plate
Add Markers from:
Existing Plate...
Marker Manager...
Assay Information File...
Marker
Detectors
Task
Create Marker...
Clear Selected
Add document to automation queue
<Back
Next>
Finish
Cancel

5. Locate the AIF on the CD (the file name is "DME_SNP_XXXXX.txt", where "XXXX" is the order number) and click **Import**.

   The software creates a marker and the appropriate detectors from the information in the AIF for each assay in your order. (In the figure below, the AIF contained only one assay, so there is only one row in the Markers to Use in Plate table.)

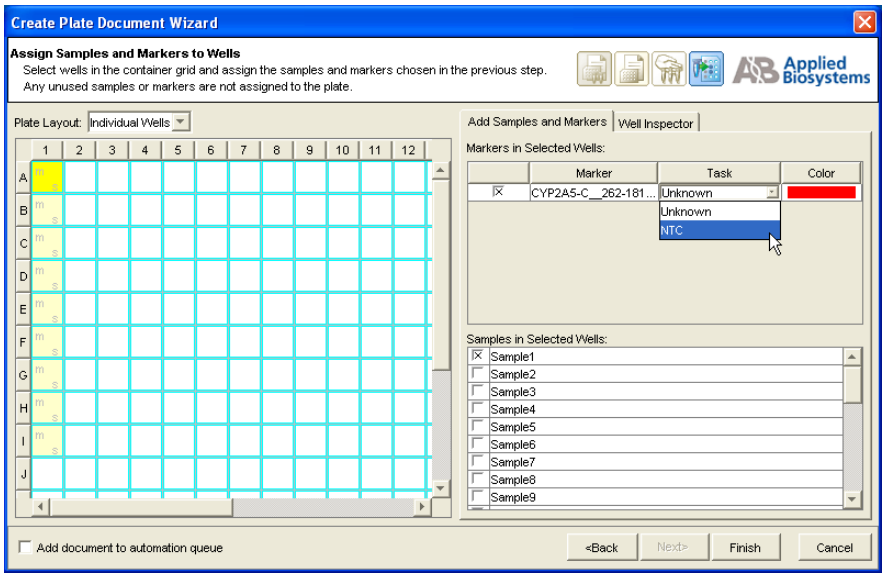


6. In the Samples table, enter the names of the samples for this plate, then click **Next**.



7. Click the well then check the appropriate sample in the Samples in Selected Wells table to assign the samples to the wells. Repeat until all the samples are assigned to wells.

8. Click the wells that contain the NTCs to select them, click the check box to the left of the assay to assign a marker to the wells, and then select **NTC** from the Task list to assign the task to the wells.



Repeat the step above for the wells containing the unknowns but assign them the **Unknown** task.

9. Click **Finish** and then run your plate.

Detectors, Markers and Tasks for 7300/7500/7500 Fast Real-Time PCR Systems

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

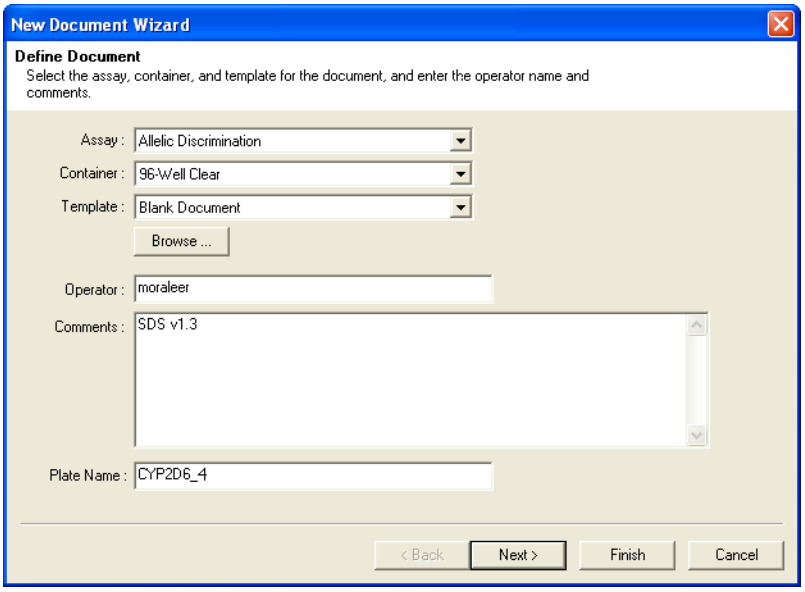
For More Information

See the *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Allelic Discrimination Getting Started Guide* (PN 4347822 Rev. C).

Create a Plate Document, the Detectors and the Marker

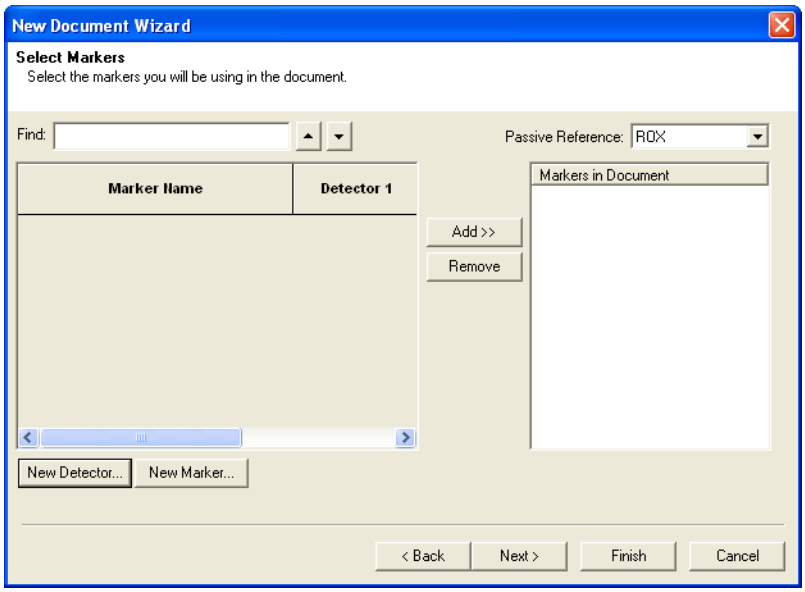
Follow the steps below to create a new plate document with new markers and detectors for an assay for the allele CYP2D6*4.

1. Open the New Document wizard by selecting **File ▸ New**.



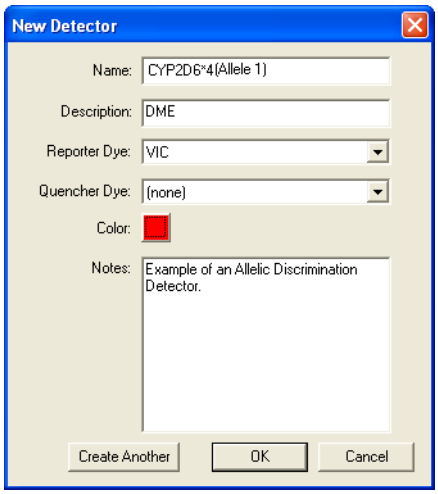
2. In the wizard:
   a. Enter the appropriate information for your study, such as the plate name.
   b. Choose **Allelic Discrimination** from the **Assay** list.

c. Click **Next** to open the Select Marker page.



3. Define the detectors for the assay. You need one detector for each allele.

a. Click **New Detector** to open the New Detector dialog box.



b. In the New Detector dialog box, for Name type **CYP2D6*4 (Allele 1)** (or the name of Allele 1).

Note: The names you assign to the detectors are displayed on the axes of the Allelic Discrimination plot in results and listed in the Call column in reports. It is good practice to assign the actual allele names to the detectors.

c. Leave the Reporter Dye set to **VIC** and the Quencher Dye set to **None**.

d. Click the color button, select a color, then click **OK**. (In this example, the color is red.)

e. Click **Create Another**.

f. For Name, type **CYP2D6*4 (Allele 2**) (or the name of Allele 2).

g. Select **FAM** for the Reporter Dye.

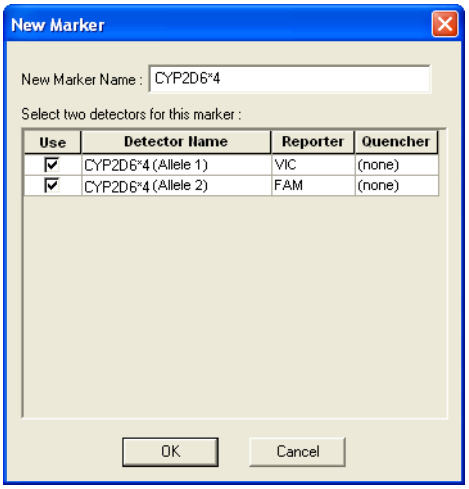
Note: Select different Reporter Dyes for the detectors. A marker (which you create next) cannot contain detectors with the same Reporter Dye.

h. Click the color button, select a color, then click **OK**. (In this example, the color is blue.)

4. Define the new marker.

a. Click **New Marker** to open the New Maker dialog box.

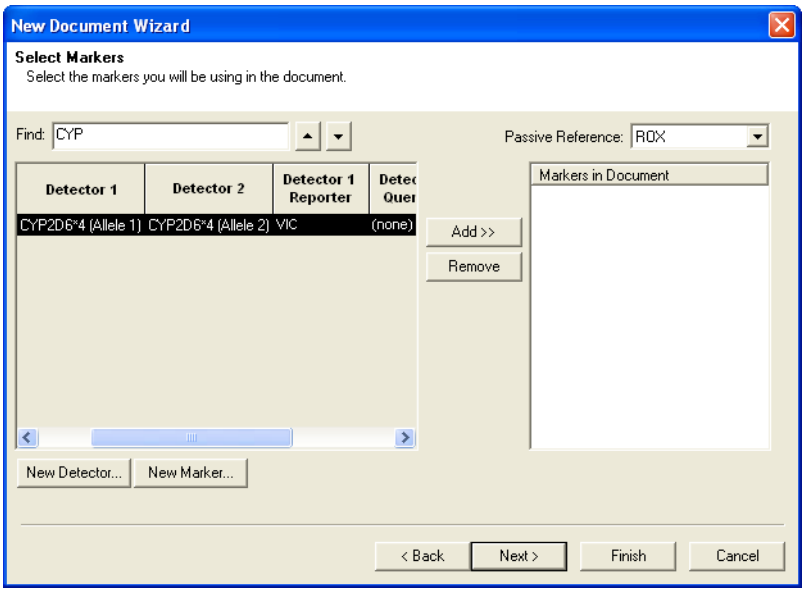
b. In the Create Marker dialog box, in the **Name** field type **CYP2D6*4** (or the marker name for your assay).



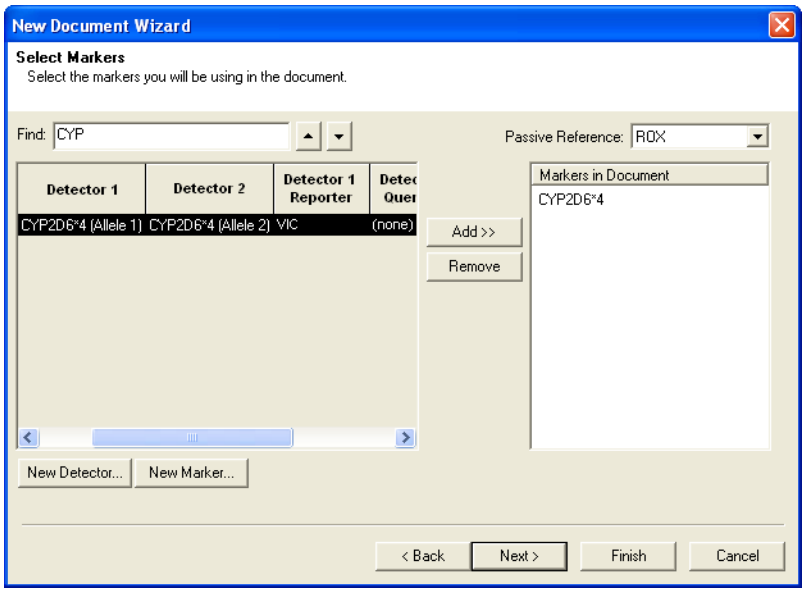
c. Click **Use** next to the **CYP2D6*4 (Allele 1)** and **CYP2D6*4 (Allele 2)** detectors you created above.

d. Click **OK**.

The new marker and its associated detectors appear in the list of markers in the New Document wizard.



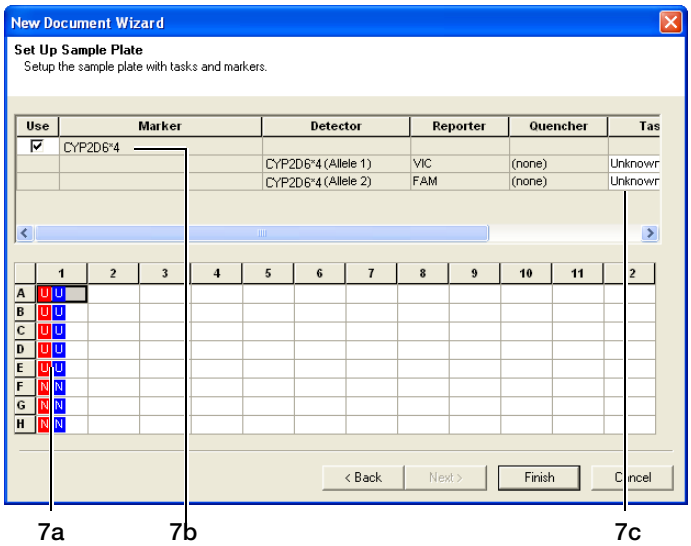
5. In the Select Markers page, click **CYP2D6*4** in the list of markers and then click **Add>>**.



The marker is added to the document.

6. Click **Next>**.

7. In the Set Up Sample Plate page, indicate the wells containing your samples, and then select the marker and assign the tasks.



   a. Click-drag to select the wells containing the NTCs.

   b. Select the **Use** box for the marker.

   c. Click the Task field for one of the detectors, then select **NTC** for task.

   d. Select the remaining wells that contain samples for this assay.

   e. Select the **Use** box for the marker. Leave the Task set to **Unknown**.

8. Click **Finish**.

Part II

Troubleshooting Using the Allelic Discrimination Plot

3

Using the Allelic Discrimination Plot

Problems with sample preparation, running the assay, the instrument, and/or software can give rise to atypical results in the allelic discrimination plot. The genetic characteristics of the assay and/or your sample can also lead to unexpected results in the allelic discrimination plot. This chapter provides pictures of atypical allelic discrimination plots and a list of possible causes, with a reference for more information.

Note: You should always follow the *TaqMan® Drug Metabolism Genotyping Assay Protocol* (PN 4362038 Rev. A).

This chapter covers:

What is a Good Allelic Discrimination Plot? 3-2
Unexpected Patterns in AD Plots 3-3

What is a Good Allelic Discrimination Plot?

An allelic discrimination plot, also known as a “cluster plot” or an “AD plot” is shown in Figure 3-1. Ideally these plots show three clusters and, near the origin, the No Template Controls (NTC). These clusters are described in Table 3-4. The points in each cluster are grouped closely together and each cluster is located well away from the other clusters.

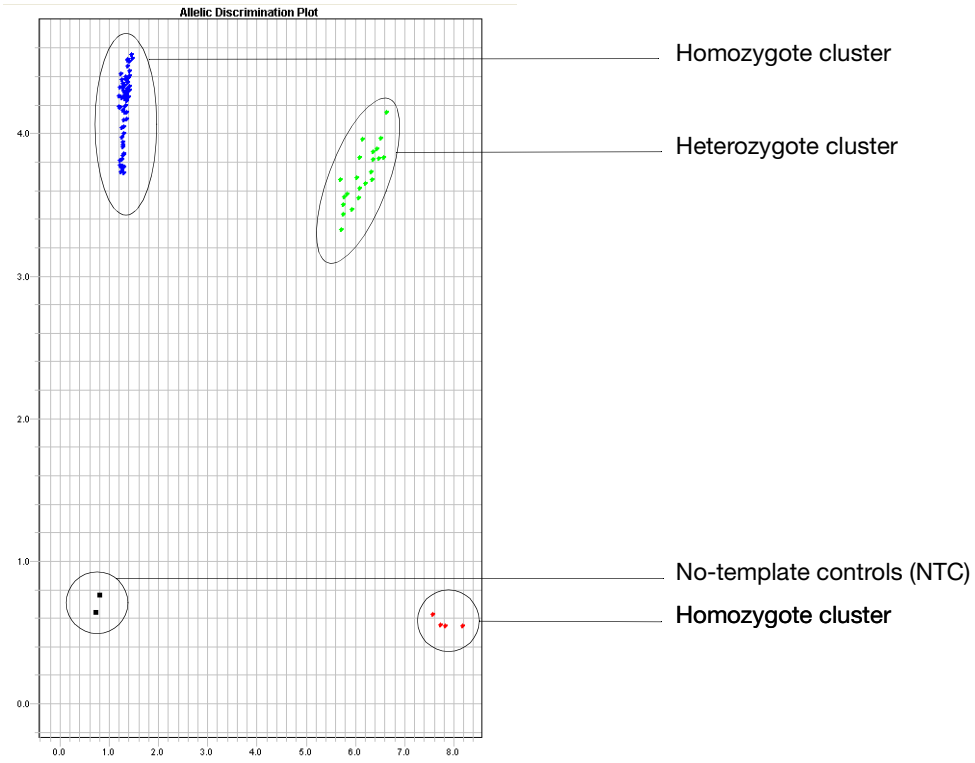


Figure 3-1 A typical three-cluster allelic discrimination plot

Table 3-4 Assignment of clusters in an allelic discrimination plot

Samples Containing...	Are Grouped In...
Allele X (homozygote), labeled with VIC® dye	Lower right corner of the plot
Allele Y (homozygote), labeled with FAM™ dye	Upper left corner of the plot
Both (Allele X and Allele Y - heterozygote)	Approximately midway between the Allele X and Allele Y clusters
No Template Control (NTC)	Bottom left corner of the plot
Undetermined	Anywhere on plot

Unexpected Patterns in AD Plots

Sometimes your allelic discrimination plot does not have the expected three cluster pattern. Genetic issues, sample preparation issues, and/or assay issues may cause unexpected patterns in allelic discrimination plots. Not every unexpected pattern is a problem. In particular, there are legitimate genetic reasons for AD plots to have other than three clusters.

Table 3-5 shows unexpected patterns you may observe in allelic discrimination plots and page numbers in this manual where those patterns and their causes are discussed.

Table 3-5 Troubleshooting from the allelic discrimination plot

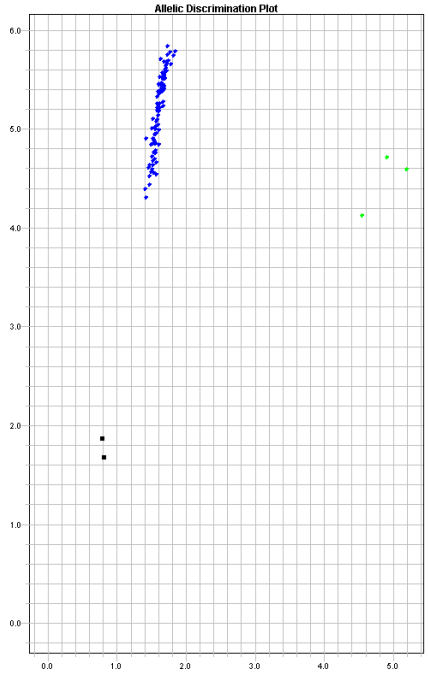
Example	Possible Causes	See Page
Only one or two clusters present	Genetic Reasons	
	Minor allele frequency (MAF) is too low for sample size from the tested population	4-2

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

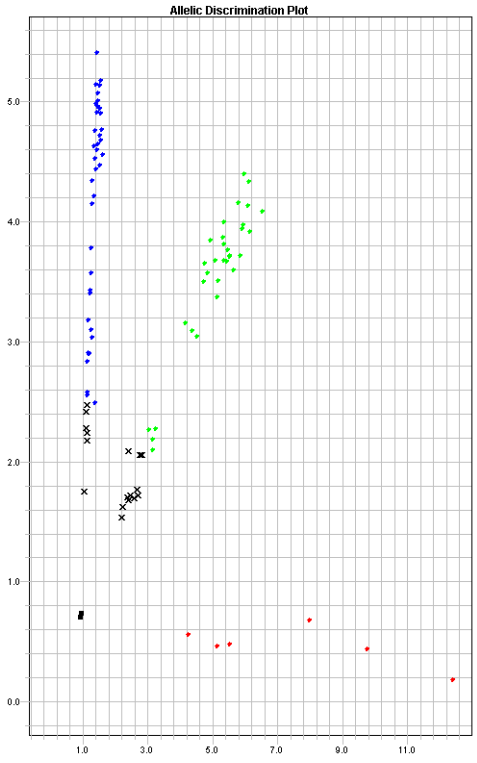
Example	Possible Causes	See Page
Trailing clusters 	Sample Preparation Problems	
	Samples not in equal quantity due to: • Degraded DNA • DNA incorrectly quantitated	 5-2 5-6
	PCR inhibitors in sample	5-5
	Assay Problems	
	Reagents mishandled or expired	5-9
	ROX™ dye not present in PCR Master Mix	5-10
	Evaporation from the sample well	5-11
	Pipetting errors	5-11
	Inefficient mixing and/or insufficient centrifugation	5-12
	Instrument Problems	
	Thermal cycler poorly calibrated	6-3
	Software Problems	
	ROX dye not designated as the reference dye	7-10

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

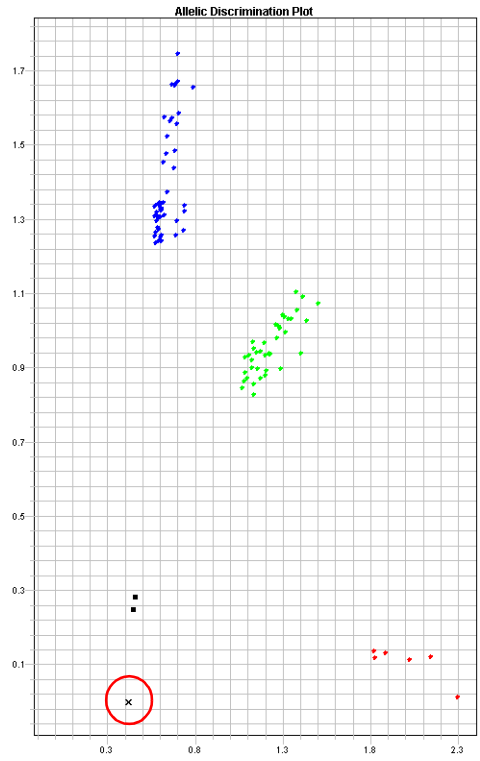
Example	Possible Causes	See Page
Some samples cluster with the NTCs 	Genetic Reasons	
	Individual sample has two null alleles	4-4
	SNP is triallelic	4-9
	Sample Preparation Problems	
	Samples not in equal quantity due to: • Degraded DNA • DNA incorrectly quantitated	5-2 5-6
	PCR inhibitors in sample	5-5
	Assay Problems	
	Evaporation from the sample well	5-11
	DNA or reagent not added to the well	5-10
	Insufficient DNA added to the well	5-11
	Pipetting errors	5-11
	Inefficient mixing and/or insufficient centrifugation	5-12
	Instrument Problems	
	Block contaminated	6-2

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

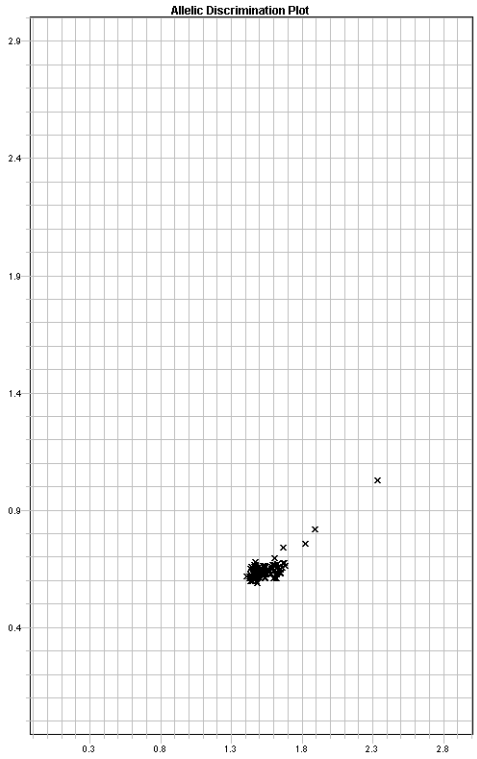
Example	Possible Causes	See Page
All samples cluster with the NTC 	Sample Preparation Problems	
	Samples not in equal quantity due to: • Degraded DNA • DNA incorrectly quantitated	 5-2 5-6
	PCR inhibitors in sample	5-5
	Assay Problems	
	Reagents mishandled or expired	5-9
	DNA or reagent not added to the well	5-10
	Insufficient DNA added to the well	5-11
	Pipetting errors	5-11
	Instrument Problems	
	Annealing temperatures on the thermal cycler were too high or too low for the primers or probes due to: • Thermal cycler poorly calibrated	 6-3
	AmpliTaq Gold® DNA polymerase was not activated efficiently, due to: • Using incorrect thermal cycler method • Thermal cycler poorly calibrated	 2-4 6-3

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

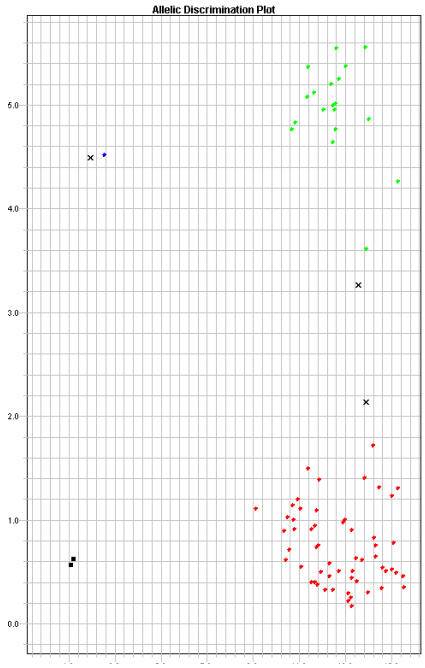
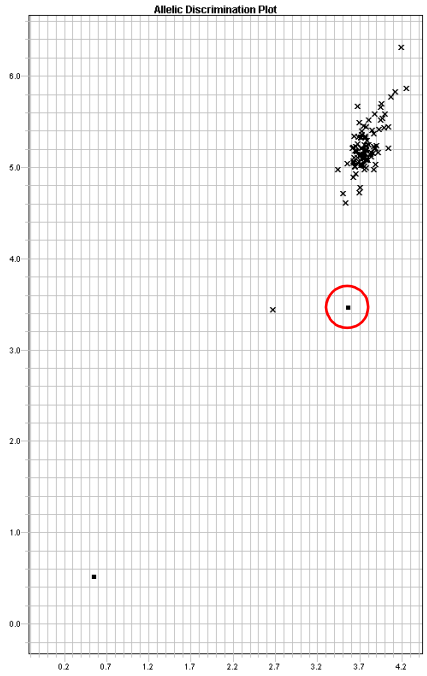
Example	Possible Causes	See Page
Cloudy or diffuse clusters 	Sample Preparation Problems	
	Samples not in equal quantity due to: • Degraded DNA • DNA incorrectly quantitated	 5-2 5-6
	PCR inhibitors in sample	5-5
	Assay Problems	
	ROX™ dye not present in PCR Master Mix	5-10
	Evaporation from the sample well	5-11
	Pipetting errors	5-11
	Software Problems	
	ROX dye not designated as the reference dye	7-10
	If only one cluster is present, allelic discrimination plot incorrectly scaled	7-8
NTCs generate high fluorescence signals that cluster with samples rather than close to the origin 	Assay Problems	
	Reagents mishandled or expired	5-9
	Contamination due to poor laboratory practices	2-2
	Software Problems	
	Reporter dye assigned incorrectly	7-15
	Instrument Problems	
	Block contaminated	6-2

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

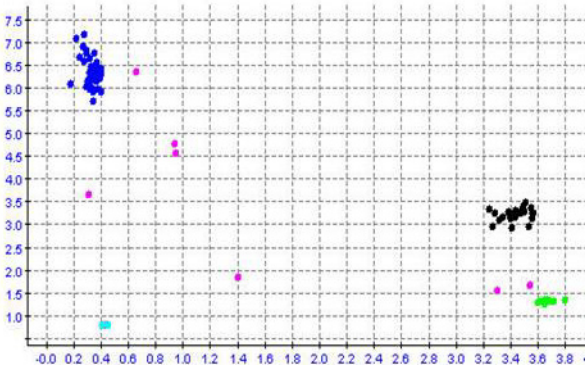
Example	Possible Causes	See Page
Sample (or samples) did not cluster with specific allele.  **Note:** In this plot: • Homozygotes are blue and green • Heterozygotes are black • NTCs are light blue • Outliers are pink	Genetic Reasons	
	Additional SNP under primer	4-5
	SNP is triallelic or tetrallelic	4-9
	Copy number polymorphism	4-7
	Sample Preparation Problems	
	Samples not in equal quantity due to: • Degraded DNA • DNA incorrectly quantitated	5-2 5-6
	Assay Problems	
	Contamination due to poor laboratory practices	2-2
	Evaporation from the sample well	5-11
	Pipetting errors	5-11
	More than one sample in the well	5-13
	Inefficient mixing and/or insufficient centrifugation	5-12
	Instrument Problems	
	Block contaminated	6-2

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

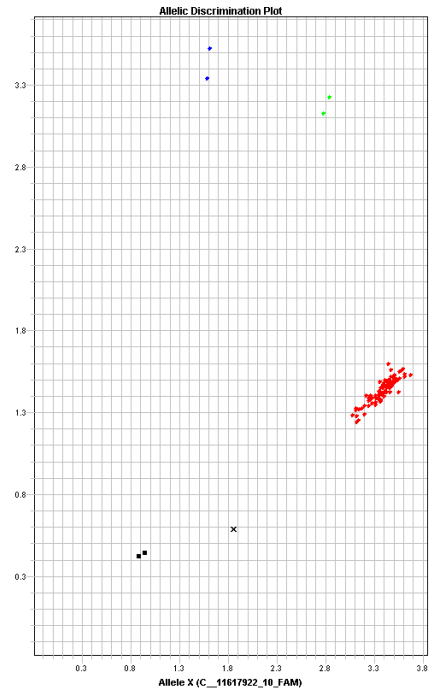
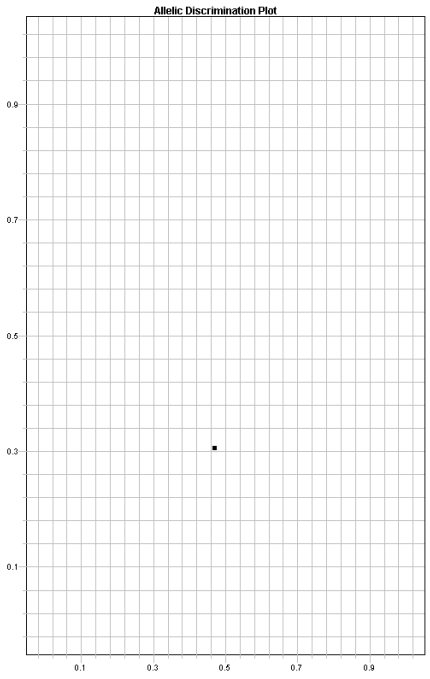
Example	Possible Causes	See Page
Samples not in Hardy-Weinberg equilibrium (expected ratios of each genotype not seen) 	Genetic Reasons	
	Copy number polymorphism	4-7
	Gene is on X chromosome	4-11
	Software Problems	
	Detectors and markers set up incorrectly	7-15
Some (or all) data is missing (no data point shown on allelic discrimination plot) 	Software Problems	
	No marker assigned to sample	7-2
	May have checked Omit for the missing well(s)	7-3

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

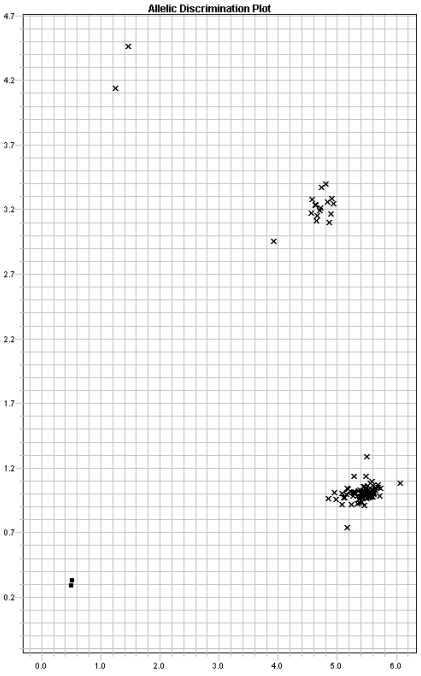
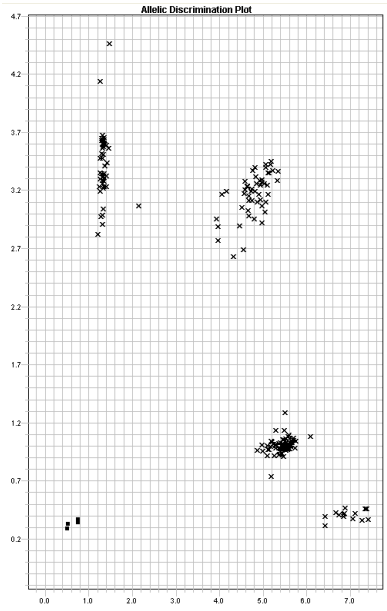
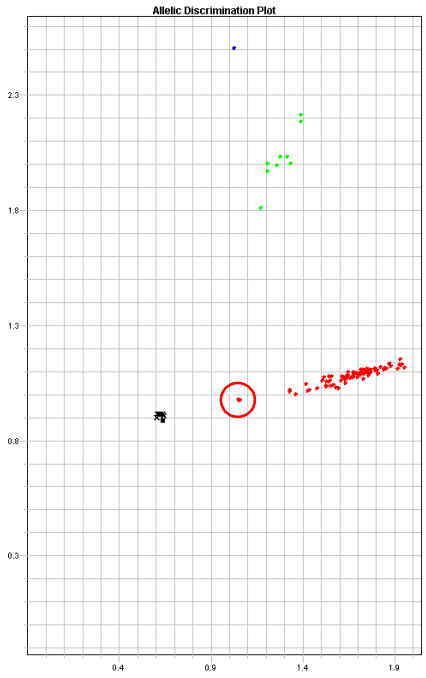
Example	Possible Causes	See Page
Some (or all) alleles not called (X is shown on the allelic discrimination plot) 	Software Problems	
	NTC task not assigned to NTC wells	7-14
	Autocall option not selected	7-4
	Sample only has two clusters, but 2-cluster calling option not selected; SDS can't assign alleles in this case	7-6
	Sample has only one cluster; SDS can't assign alleles in this case	7-8
	Outlier sample too far off scale for alleles to be called for other samples	7-12
More than three clusters 	Software Problem	
	Several assays were run but only one marker was assigned	7-21
	Genetic Reasons	
	Additional SNP under probe	4-5
	Copy number polymorphism	4-7
	SNP is triallelic or tetrallelic	4-9

Table 3-5 Troubleshooting from the allelic discrimination plot *(continued)*

Example	Possible Causes	See Page
Vector cluster (sample data has two clusters at the same angle) 	Genetic Reasons	
	Additional SNP under probe	4-5
	Sample Preparation Problems	
	Samples not in equal quantity due to: • Degraded DNA • DNA incorrectly quantitated	 5-2 5-6

4

Understanding Genetic Issues

Genetic issues my cause atypical or unexpected assay results. Use this chapter to determine reasons for unexpected or atypical genetic results.

This chapter addresses the following possible genetic causes for unexpected results:

Low Allele Frequency 4-2
Null Alleles in an Individual 4-4
Additional SNP Present Under the Probe or Primer 4-5
Gene Has a Copy Number Polymorphism 4-7
SNP is Triallelic or Tetrallelic 4-9
Gene on the X Chromosome 4-11

Low Allele Frequency

Only one or two clusters can occur in the allelic discrimination plot when the minor allele occurs at a very low frequency in the population being studied.

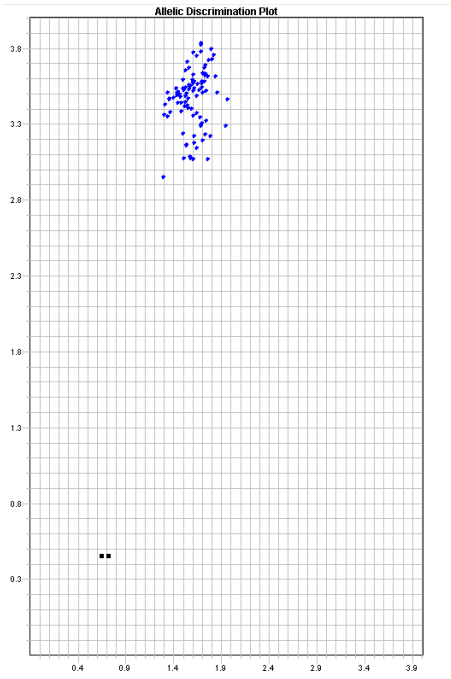


Figure 4-1 Allelic discrimination plot showing a single cluster (in addition to the NTCs)

What to Do

To determine if the size of your sample population is large enough to detect the minor allele of interest:

1. Find the Minor Allele Frequency (MAF) for your assay on the TaqMan® Drug Metabolism Genotyping Assays page at the Applied Biosystems web site at **dme.appliedbiosystems.com**, which is frequently updated.

   Alternatively, find the MAF in the Assay Information File distributed with your assays. You might also find allele frequency data using the public SNP identifier, from public web sites such as:

   - dbSNP at **www.ncbi.nlm.nih.gov/SNP/index.html**
   - the HapMap project at **www.hapmap.org**

2. Using the Hardy-Weinberg Equilibrium equation, determine if the minor allele is detectable for a sample the size of your test population (see "Example Calculation" on page 4-3).

   In the Hardy-Weinberg Equilibrium equation, $q^2 + 2qp + p^2 = 1$, the expected genotype frequencies are q^2, $2qp$, and p^2, where q and p represent the allele frequencies.

   The values for q^2, 2qp, and p^2 correspond to the fraction of a given population that would be homozygous for the minor allele (qq), heterozygous (qp), and homozygous for the major allele (pp), respectively.

3. Multiply your sample size by the fraction for each allele to determine the number of individuals with each genotype that you should expect to see.

   If your sample size is small, you may not be able to detect rare alleles.

Example Calculation

For a SNP with a MAF of 5% (0.05), the predicted frequencies are 0.0025 q:q, 0.095 q:p, and 0.9025 p:p.

If you test of 20 genomic DNA samples from this population, you might expect:

- Homozygotes for the minor allele – 0.0025 × 20 = 0.05, no individuals
- Heterozygotes – 0.095 × 20 = 1.9, about 2 individuals
- Homozygotes for the major allele – 0.9025 × 20 = 18.05, about 18 individuals

To detect one homozygote for the minor allele, it would take a sample size of approximately 400 individuals (Sample Size = $1/MAF^2$).

Discussion

Several of the assays in the this collection show low or 0 for the minor allele frequency. Many of these SNPs are believed to be functional polymorphisms which may occur at very low frequencies, depending on the population you are studying. Many functional polymorphisms occur at low frequencies (Wong 2003). The MAF indicates the frequency of the less-frequent allele in a population. (Traditionally, only the minor allele frequency is reported. The major allele frequency is calculated as 1 – MAF.) From the MAF, you can calculate how large the sample population needs to be to detect a specific allele. The lower the frequency of the minor allele, the larger the sample size required to detect the allele.

The allele frequencies for the TaqMan Drug Metabolism Genotyping Assays were calculated for four populations:

- African American
- Caucasian
- Chinese
- Japanese

Some of the assays are for polymorphisms that may:

- Not occur in some populations

  or

- Have very low minor allele frequencies

An example is assay ID C__11703892_30 for gene ALDH2 (rs671). In the Caucasian/African American test populations, the minor allele frequency was 0 but in the tested Chinese/Japanese populations it was 20-30%.

During development of the TaqMan Drug Metabolism Genotyping Assays, a sample size of 45 people was tested in each of the four populations. This sample size provides 95% confidence that alleles with a minor allele frequency of 5% will be detected. Therefore, the sample sizes of the populations tested for the DMEs were too small to detect allele frequencies less than 5%.

Null Alleles in an Individual

When an individual does not have the gene or the portion of the gene that contains the SNP of interest, the individual has a "null allele." The data point in the allelic discrimination plot from such an individual will either:

- Appear as a homozygote of the allele that is present (where there is one null allele).

  or

- Cluster with the NTCs (when there are two null alleles).

If an individual sample consistently clusters with the NTCs for a particular assay, it may indicate the individual has a null allele.

There are documented occurrences of null alleles in the genes CYP2A6 (Topcul, 2002), GSTM1 (Smits, 2003), GSTT1 (Bolt, 2006, Thier, 2006, Cho, 2005, and Rebbeck, 1997), and CYP2D6 (Luo, 2005, Zanger, 2004) in the TaqMan® Drug Metabolism Genotyping Assays collection.

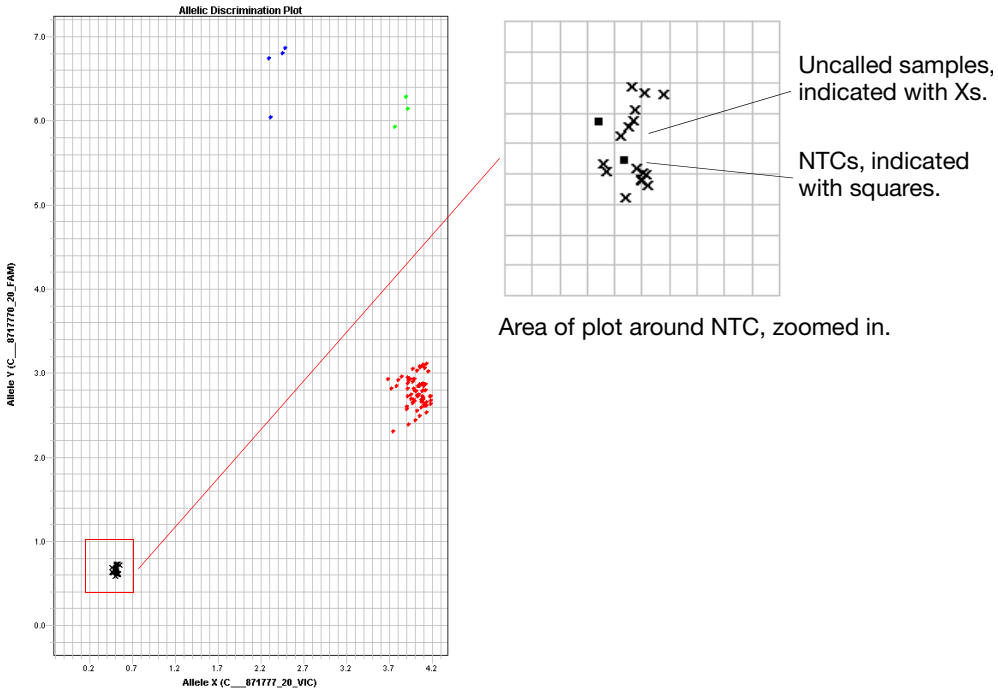

Allelic Discrimination Plot
Allele Y (C__8717770_20_FAM)
Allele X (C__8717777_20_VIC)
Uncalled samples, indicated with Xs.
NTCs, indicated with squares.
Area of plot around NTC, zoomed in.

Figure 4-2 Allelic discrimination plot showing a null allele for the assay C__8717770_20

What to Do

1. Evaluate the overall assay performance:
   - Do the assay results appear in tight clusters?
   - Do the clusters have good separation?
2. Repeat the experiment. If the same sample(s) consistently cluster with the NTC while other samples show fluorescence, a null allele may be present.

3. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
4. Perform a literature search for documentation reporting the presence of null alleles for the gene.
5. To rule out assay interference caused by a SNP present in the individual's DNA, perform comparative sequencing on the subjects to identify any undocumented SNPs.
6. Perform a TaqMan® Gene Copy Number Assay (PN 4331182) on all samples to confirm your sample has a null allele.‡

Additional SNP Present Under the Probe or Primer

A non-target SNP under a primer or probe may result in off-cluster data. The location of the non-target SNP under the primer or probe, as well as the MAF, influences the extent to which the cluster pattern is atypical. The number of individuals exhibiting this pattern depends on the allele frequency of the non-target SNP.

You may see additional clusters ("angle clusters") or a lack of amplification of the sample when there is an additional polymorphism under the primer; see Figure 4-3 on page 4-5. The presence of a polymorphism under a primer generally leads to lower PCR efficiency.

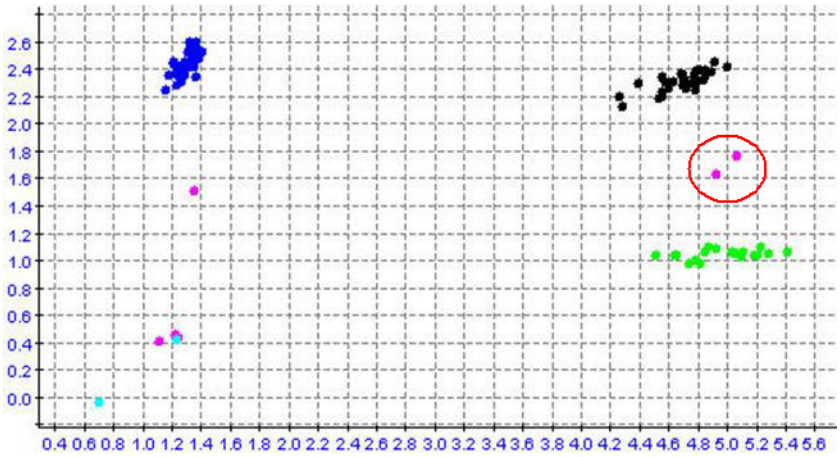


Figure 4-3 SNP under primer. The points in pink between the black and green cluster constitute an angle cluster. (Note that this example is not a TaqMan® Drug Metabolism Genotyping Assay but is included here for illustration.)

A SNP under a probe can result in an outlier that falls between the heterozygote and one of the homozygotes (an angle cluster) or an outlier that has the same angle as a cluster but trails behind the main cluster (a vector cluster).

‡ The TaqMan Gene Copy Number Assays are part of the TaqMan® Gene Expression Assay product line. To locate the copy number assay of interest, in the Choose Set Membership section of the search page at the Applied Biosystems web site, click **Search Gene Copy Number Assays**.

You may see points that are in agreement with a cluster but trail behind the main cluster (a “vector cluster”) when there is an additional SNP under the probe; see Figure 4-4. The presence of a SNP under a probe leads to lower fluorescence intensity.

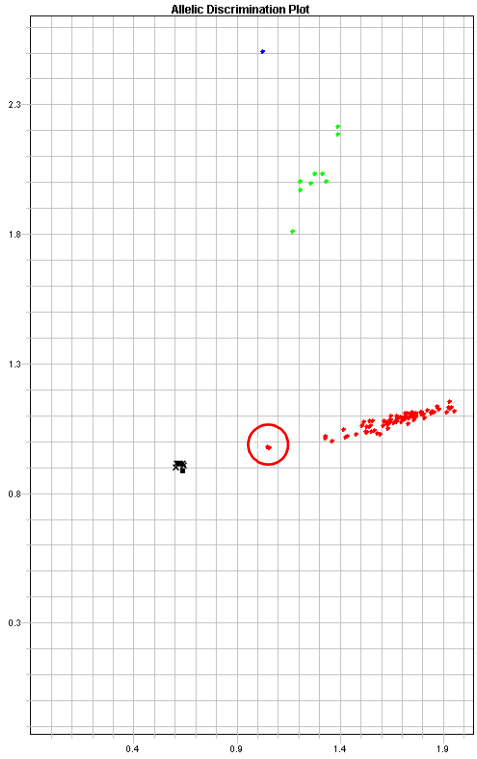


Figure 4-4 SNP under probe circled in red. The red cluster is a vector cluster. (Note that this assay is not a TaqMan® Drug Metabolism Genotyping Assay, but is included here for illustration.)

What to Do

To confirm the presence of another SNP under the probe or primer:

1. Repeat the experiment and evaluate overall assay performance.
   - Do the assay results appear in tight clusters?
   - Do clusters have good separation?
2. Verify the presence of the outlier.
3. Examine the sample’s performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
4. Search the public databases, such as dbSNP, to see if the additional SNP has been discovered.
5. Perform comparative sequencing on the subjects to identify any undocumented SNPs present under the primer or probe. The presence of extra SNPs may cause angle clusters or vector clusters.

Discussion

The Applied Biosystems assay design process included many checks to assure that primers and probes were not designed over polymorphisms other than the intended SNP target. However, the growing number of SNPs discovered in studies of different ethnic populations make it likely that some of the primers and/or probes in the TaqMan Drug Metabolism Genotyping Assays may overlap currently unknown polymorphisms in certain populations.

In some rare cases, there are some assays where primers and probes are located over SNPs or other polymorphisms due to the close proximity of the two SNPs. For these assays, the vector cluster falls in line with samples of the same genotype, but the reduced PCR efficiency causes a reduction in signal intensity.

Gene Has a Copy Number Polymorphism

A copy number polymorphism for a gene may or may not appear as an anomaly in the allelic discrimination plot.

- If an individual is homozygous with more than three copies of the gene and each copy has the same genotype, the data will most likely appear in the homozygous cluster.
- If an individual is heterozygous with an odd number of copies and the copies have different genotypes, then the data will probably fall between the clusters for the heterozygote (T:A) and the homozygote (A:A).

Several of the genes included in the TaqMan Drug Metabolism Genotyping Assays are known to have copy number polymorphisms: CYP2D6 (Ouahchi, 2006 and Wilkinson, 2005), GSTM1 (Ouahchi, 2006), GSTT (Ouahchi, 2006), CYP2E1 (Liew, 2005), and CYP2A6 (Ouahchi, 2006, Oscarson, 2001, Rao, 2000 and Xu, 2002).

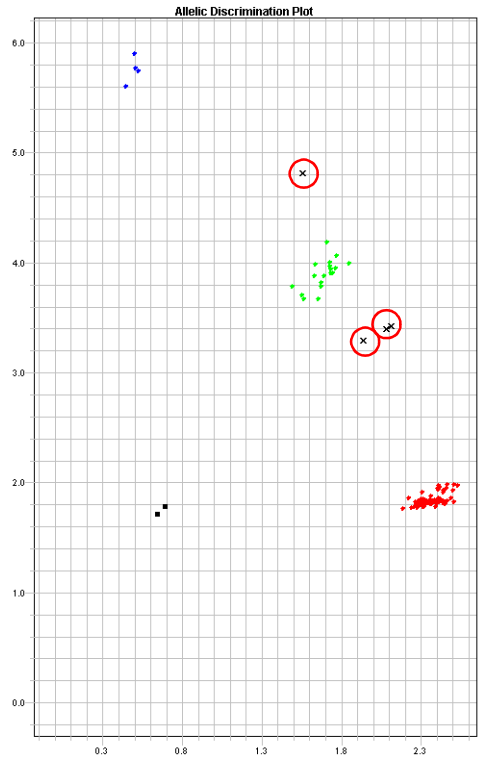


Figure 4-5 Allelic discrimination plot for CYP2D6, showing samples with a copy number polymorphism (circled in red) for assay C__32407252_30

What to Do

1. Evaluate overall assay performance
   - Do the assay results appear in tight clusters?
   - Do clusters have good separation?
2. Repeat the experiment to confirm the presence of the off-cluster sample.
3. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
4. Perform a literature search for documentation of copy number polymorphisms for the gene.
5. Perform comparative sequencing on the subjects to identify any undocumented SNPs present under the primer or probe; extra SNPs may cause angle clusters.
6. Perform a TaqMan Gene Copy Number Assay (PN 4331182) on all samples to determine the copy number for the gene in which the polymorphism resides.‡

‡ The TaqMan Gene Copy Number Assays are part of the TaqMan Gene Expression Assay product line. To locate the copy number assay of interest, in the Choose Set Membership section of the search page at the Applied Biosystems web site, click **Search Gene Copy Number Assays**.

Discussion

Data points for samples from homozygous individuals with extra copies of a gene will generally cluster with the homozygous cluster. Data points for heterozygous individuals with copy number polymorphisms may appear as outliers such as a fourth or fifth cluster between the heterozygote cluster and one of the homozygous clusters. Since copy number variation may not present itself in all individuals, a gene dosage assay should be performed on all samples to determine which individuals carry extra copies of the gene.

SNP is Triallelic or Tetrallelic

When a SNP is triallelic or tetrallelic, you may see outlier samples in the allelic discrimination plot, although the samples may not be well separated from the main clusters. You may also see more than three clusters. These situations are best confirmed by running replicate plates. Applied Biosystems did not include known triallelic SNPs in the TaqMan Drug Metabolism Genotyping Assays collection.

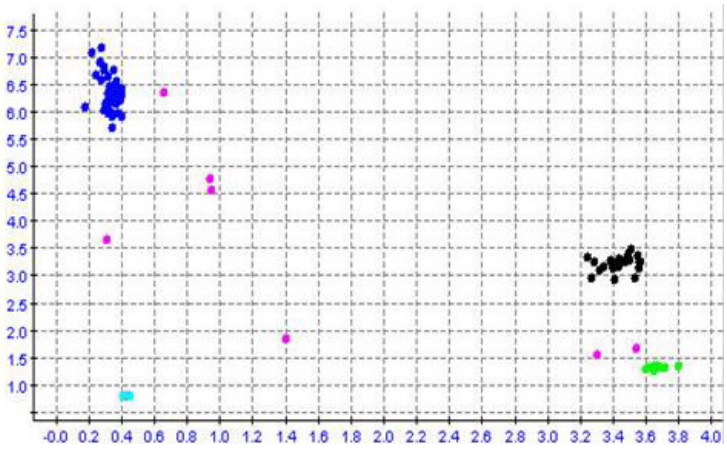


Figure 4-6 Allelic discrimination plot for a triallelic gene; additional clusters shown in pink. (Note that this assay is not a TaqMan® Drug Metabolism Genotyping Assay, but is included here for illustration.)

What to Do

1. Evaluate overall assay performance: Are there consistent outlier samples?
2. Examine the sample's performance in other assays to rule out problems caused by this particular sample, such as sample impurity or degradation.
3. Perform comparative sequencing on the subjects to verify the presence of more than two alleles.
4. Repeat the experiment. If the same samples are consistently located in the same outlier space (away from the NTCs, the heterozygotes and the homozygotes) your gene may be triallelic.
5. Check the literature for the SNP in question. There may be newly reported polymorphisms described in the literature. Calculate the allele frequencies for your plate and compare them to the literature to confirm your results agree with the literature.

Discussion

If a SNP is triallelic, you might see six clusters (three homozygotes and three heterozygotes) rather than the typical pattern of three clusters (two homozygotes and one heterozygote). If a SNP is tetrallelic, the possible cluster pattern can be more complicated. Figure 4-6 shows an assay created for the SNP rs2032582 in the ABCB1 gene which is known to have all four alleles in several populations.

In the triallelic example in Figure 4-7, the following alleles are present:

Table 4-5 Possible genotypes in a triallelic gene

Bases in DNA	Possible Homozygotes	Possible Heterozygotes
Original gene: G, T	GG and TT	GT
Triallelic gene: G, T, A	GG, TT, AA	AT, GT, GA

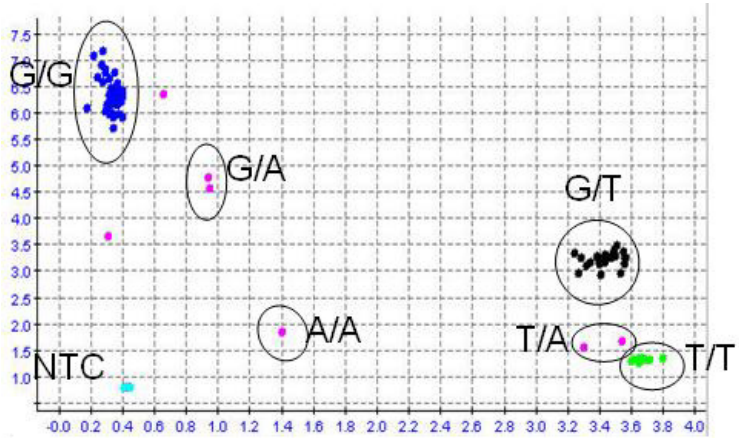


Figure 4-7 Triallelic with the genotype of each cluster shown

Gene on the X Chromosome

When a gene is on the X chromosome and the population being studied is made up of both males and females, the genotype frequencies of the samples do not correspond to the predicted autosomal Hardy-Weinberg frequencies. For a sample population composed of a mixture of males and females, the number of heterozygotes will be noticeably lower than predicted by the Hardy-Weinberg equilibrium equation. None of the males should be heterozygous because males have only one X chromosome.

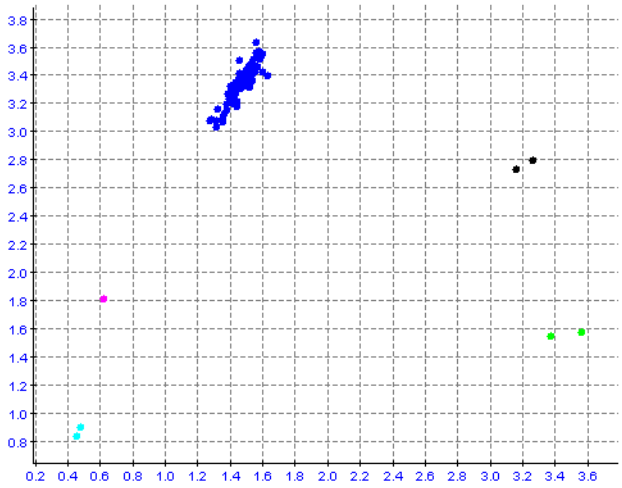


Figure 4-8 Allelic discrimination plot with a small number of heterozygotes for assay C__11617922_10 for SNP rs6324 in the MAOB gene

What to Do

1. Check the AIF included with the assay or the TaqMan Drug Metabolism Genotyping Assays page at the Applied Biosystems web site at **dme.appliedbiosystems.com** to determine if the assay is for a target located on the X chromosome.
2. Check your results by gender.

Discussion

When a SNP is located on the X chromosome, only the females in the population can be heterozygous. Males, with only one X chromosome, will always be homozygous. Depending upon the minor allele frequency, you may see males in only one of the two homozygous forms.

Figure 4-9 on page 4-12, shows the results from Figure 4-8 color-coded by gender, with the males shown in brown. Note there are no male heterozygotes.

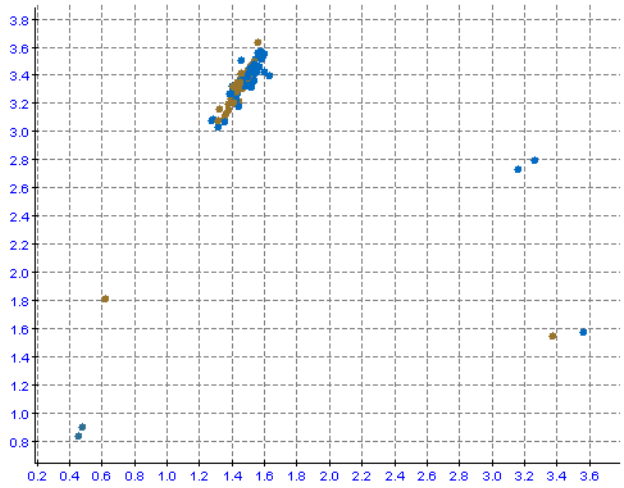


Figure 4-9 Same data from Figure 4-8, with data points colored by gender: blue – female, brown – male

5

Sample Preparation and Assay Problems

This chapter discusses problems in sample and assay preparation and remedies for those problems.

This chapter covers:

Sample Preparation Problems 5-2
Assay Problems 5-9

Sample Preparation Problems

Problems with preparing the genomic DNA for the assay include:

- "Degraded DNA" on page 5-2
- "PCR Inhibitors in Sample" on page 5-5
- "Inaccurate DNA Quantitation" on page 5-6

Degraded DNA

Degraded DNA can affect PCR efficiency due to the presence of fewer template copies, which will affect the success of your TaqMan® Drug Metabolism Genotyping Assay. Degradation can result from:

- using very old DNA samples
- using DNA extracted from formalin-fixed paraffin embedded samples
- freezing and thawing DNA samples repeatedly
- leaving DNA samples at room temperature
- exposing DNA samples to heat or physical shearing
- purifying DNA samples inefficiently so residual nucleases remain

What to Do

- Run an agarose gel to determine if your DNA is degraded. Look for a tight band of high molecular weight; smearing indicates degraded DNA. (Figure 5-1 illustrates DNA degraded by heat.)

  If the DNA is substantially degraded, use more caution in interpreting your results. If possible, consider repeating the assay using freshly prepared genomic DNA samples.
- For future experiments, follow the sample storage guidelines in Table 5-6 on page 5-4.

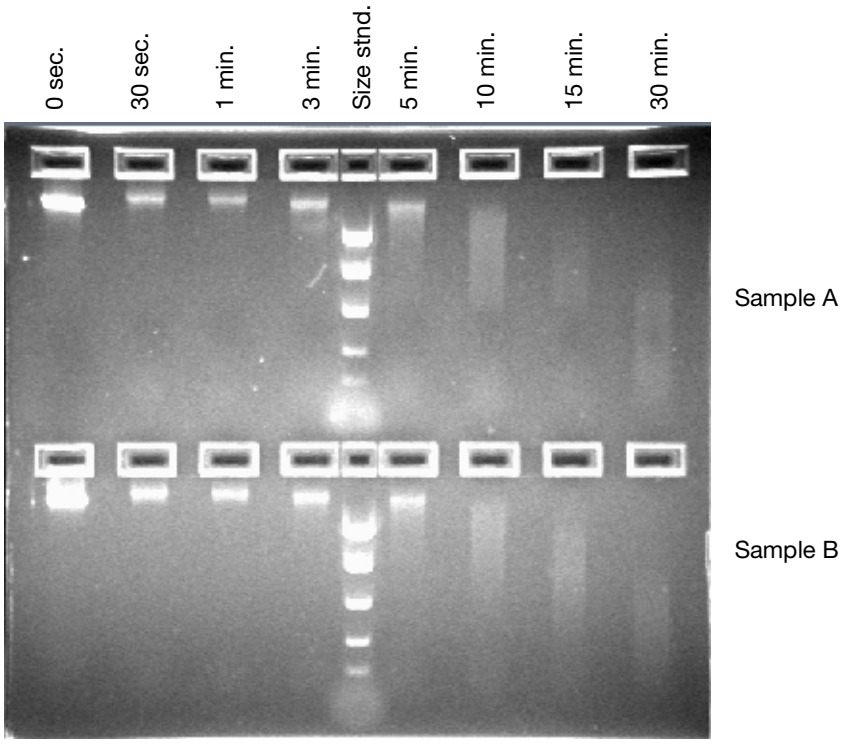


Figure 5-1 Agarose gel stained with ethidium bromide, showing two samples of human gDNA subjected to heating at 99 °C for 0 to 30 minutes. The effects of heating on a TaqMan® Drug Metabolism Genotyping Assay, for these samples and others, are shown in Figure 5-2.

Discussion

As the average size of the DNA in a degraded sample approaches the size of the target sequence, the amount of PCR product generated is reduced because there are fewer intact templates in the size range necessary for amplification. An example of degradation by heating is illustrated in Figure 5-2. You can expect similarly poor assay results for gDNA degraded by other causes.

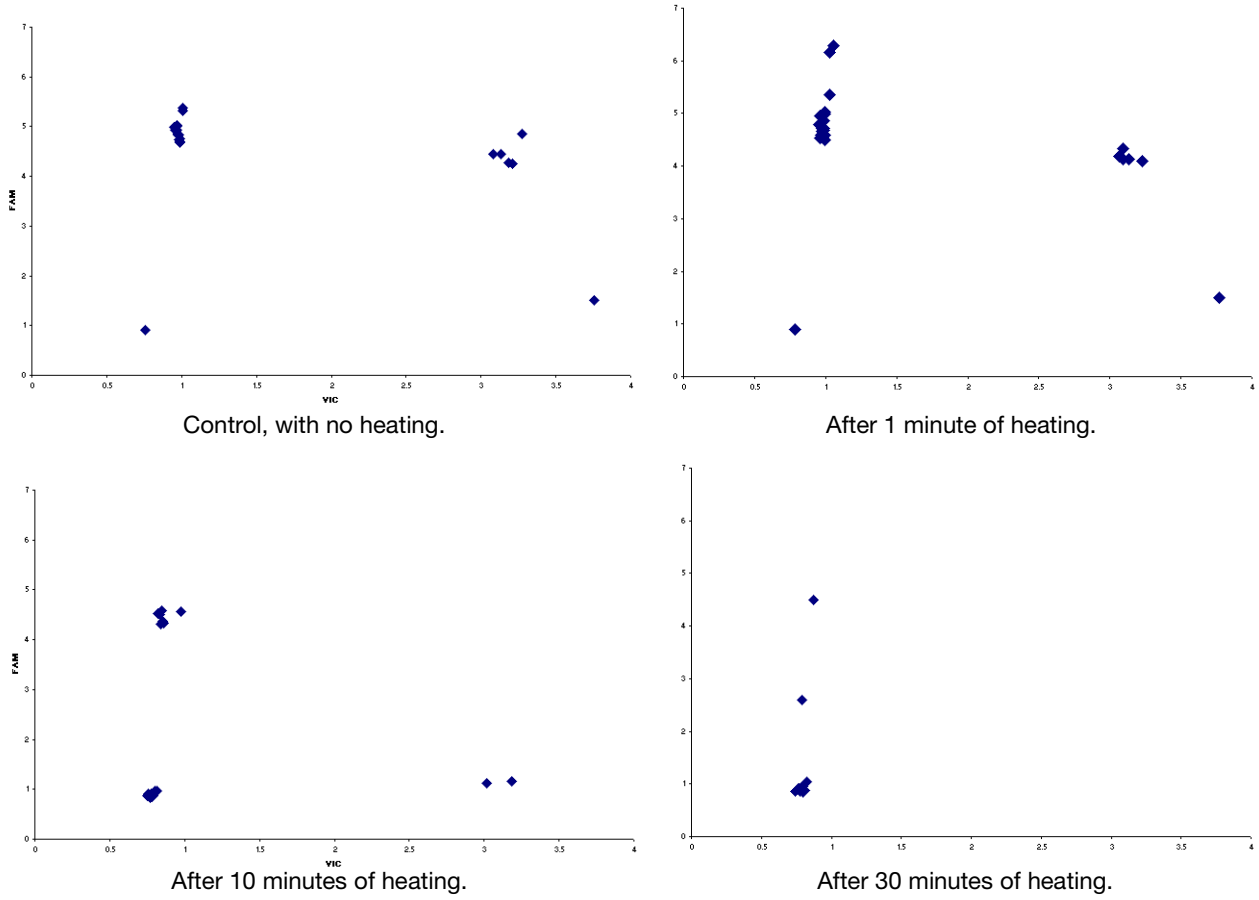


Figure 5-2 Allelic discrimination plots showing the effects of DNA degradation caused by heating

Factors that affect DNA degradation include tissue preservation methods, exposure to UV radiation, temperature, pH, and salt concentration of the environment (Dean, M. and Ballard, J.W.O., 2001). There are many sources of gDNA including fresh capillary blood, buccal scrapes, solid organ biopsies, and paraffin embedded tissue; Table 5-6 lists recommended sample storage conditions to help minimize DNA degradation.

Table 5-6 Recommended sample storage conditions

Tissue Type	Storage Conditions
Buccal tissue	• Store frozen at –15 to –25 °C.
Tissue	• Immediately place tissue in liquid nitrogen and store at –80 °C *or* • Freeze and store at –15 to –25 °C

Table 5-6 Recommended sample storage conditions *(continued)*

Tissue Type	Storage Conditions
Blood	• Whole blood: store frozen at –15 to –25 °C. • Buffy coat: frozen at –15 to –25 °C and thaw at room temperature before use.

PCR Inhibitors in Sample

Potential PCR inhibitors can originate from the tissue source of the DNA sample or from the purification method. Examples of inhibitors originating from the cell include heparin (Holodiny et al, 1991), proteins, and heme (Akane et al, 1994, DeFranchis et al., 1998). Examples of inhibitors originating from DNA preparation are phenol (Katcher and Schwartz, 1994), proteases, detergents (SDS), and salts.

The presence of polymerase inhibitors can decrease PCR efficiency, leading to:

- Trailing clusters
- Nonamplification such that some (or all) samples cluster with the NTCs

What to Do

1. Dilute the sample and run the assay with the diluted sample. If the inhibition decreases, then it is likely there are PCR inhibitors in the sample.
2. Re-purify the sample and run the assay again.

Discussion

Inhibition of PCR is always possible when DNA is extracted from tissue and/or blood samples.

Applied Biosystems examined the effects of hematin on the TaqMan Drug Metabolism Genotyping Assays. Hematin was added to each well of the assay at one of three concentrations (0.25 µM, 0.50 µM, and 1.00 µM), except for the wells containing the control samples. The results are shown in Figure 5-3.

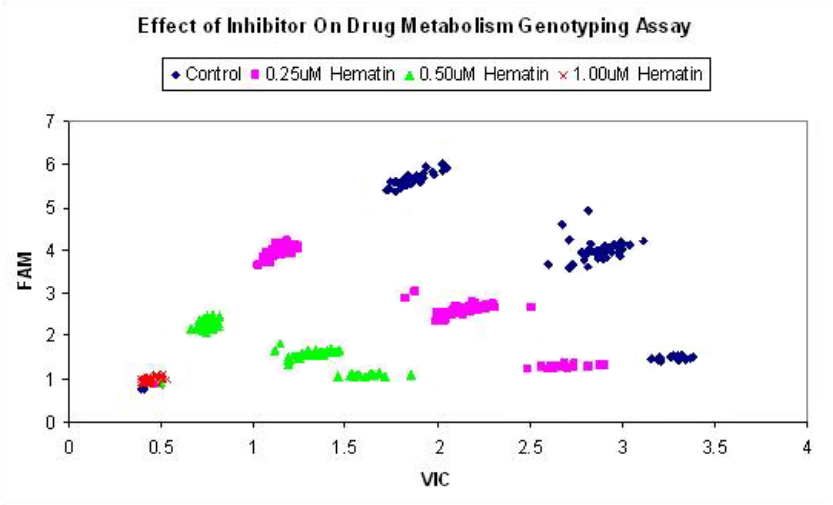


Figure 5-3 PCR Inhibition as a function of hematin concentration

PCR inhibition effects begin at 0.25 µM hematin. Assay performance is severely compromised at 0.50 µM hematin, and although signal strength is significantly lowered, it is still possible to call genotypes. Assay performance is entirely inhibited at 1.00 µM, where cleaving of the probes is nonexistent, resulting in no fluorescence.

The DNA purification method you use to prepare your DNA can affect the success of PCR (Maaroufi et al., 2004). Choose a method that minimizes degradation and removes inhibitors. One method for assessing DNA purity is to calculate the A_{260}/A_{280} ratio. In addition, absorbance at 230 nm can indicate the presence of phenol (Gallagher 1994).

In Applied Biosystems laboratories, A_{260}/A_{280} ratios between 1.8 and < 2.0, indicate that the gDNA samples are pure enough to use for TaqMan® Drug Metabolism Genotyping Assays. The effective read range of UV spectroscopy is 0.1 to 0.999, which corresponds approximately to 4ng/µL to 50ng/µL of genomic DNA. Values above or below that range are invalid absorbance readings. To ensure accurate quantitative results, gDNA samples should be diluted so that the A_{260} reading is between 0.1 to 0.999. Remember to record the dilution factor and the diluents used. Most plates and cuvettes have minimum working volumes, and the genomic DNA sample used for the quantitative measurement will be discarded. Ensure that you have enough gDNA to use this method and still leave sufficient DNA for your study.

Inaccurate DNA Quantitation

Problems with DNA quantitation manifest themselves as:

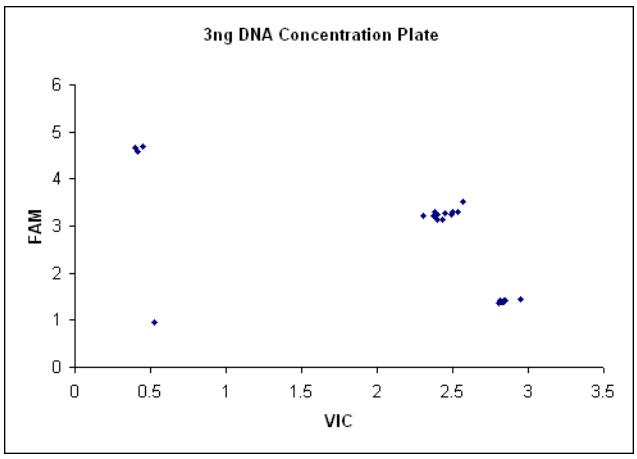
- Trailing clusters
- Some (or all) samples clustering with the NTCs
- Cloudy or diffuse clusters
- A sample (or samples) does not cluster with a specific allele (i.e. is an outlier)

What to Do

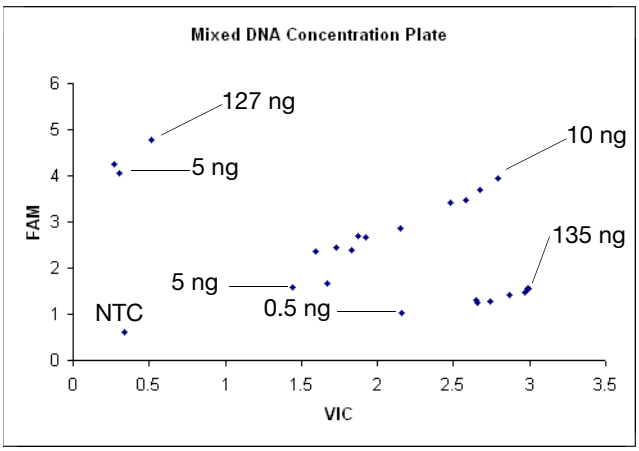
- Always perform your own concentration measurements before using any genomic DNA (gDNA), even commercially prepared DNA.
- Use the recommended amount of gDNA, 3 to 20 ng per sample per assay.
- Always use the same quantity of gDNA for all samples of an assay on a plate.

Discussion

The amount of genomic gDNA is critical to the success of the assays. Within an assay and/or study, gDNA concentration uniformity leads to accurate, robust, and reproducible results and ensures efficient use of valuable samples. Variability in gDNA concentrations can lead to experimental anomalies that may affect interpretation of genotyping results, as shown in Figure 5-4. Precise handling and quantitative measurements before running an assay can prevent possible errors without waste of reagents and samples.



All samples have 3 ng gDNA



Samples with gDNA ranging from 0.5 to 127 ng

Figure 5-4 Allelic discrimination plots for TaqMan® Drug Metabolism Genotyping Assay C_1204092_20

Commercially purchased DNA comes with concentration information, but it is good practice to confirm the DNA concentrations in your own laboratory. Applied Biosystems has found that the concentrations of DNA listed for commercially available genomic DNA can be quite different from our laboratory measurements.

There are numerous methods for quantitating genomic DNA, including:

- UV spectroscopy
- Absolute quantitation
- Fluorometric analysis

Applied Biosystems recommends quantitating genomic DNA with UV spectroscopy or absolute quantitation using the TaqMan® RNase P method.

UV Spectroscopy

UV Spectroscopy is the most widely used method for quantifying DNA of all types; however, the consumable reagents used in the process vary greatly. Ensure your spectrophotometer is set up correctly for the reagents and equipment you will use. Optical plastic cuvettes and plates have different background constants than quartz cuvettes and plates; consult the instrument's manual for ways to determine background constant. Also, be cautious of the diluents used with the gDNA samples. They too can have differing properties and may affect the final results.

UV spectroscopy can be used to quantitate gDNA by reading sample absorbance at 260 nm (A_{260}). The A_{260} is most accurate when using pure nucleic acid and is most useful for DNA in microgram quantities (Gallagher 1994). Proteins, particles in the solution, and aromatic chemicals can affect the reading. (Samples are usually concurrently read at 280 nm, to determine the concentration of contaminating proteins. The A_{260}/A_{280} ratio is used to determine purity of a DNA sample; see "PCR Inhibitors in Sample" on page 5-5.)

The effective read range of UV spectroscopy is 0.1 to 0.999, which corresponds approximately to 4ng/µL to 50ng/µL of genomic DNA. Values above or below that range are invalid absorbance readings. To ensure accurate quantitative results, gDNA samples should be diluted so that the A_{260} reading is between 0.1 to 0.999. Remember to record the dilution factor and the diluents used. Most plates and cuvettes have minimum working volumes, and the genomic DNA sample used for the quantitative measurement will be discarded. Ensure that you have enough gDNA to use this method.

Absolute Quantitation

Absolute quantitation measures the total amount of amplifiable gDNA. This technique requires the creation of a standard curve using gDNA samples of known quantities. The standard samples must be prequantitated and validated using an independent method such as spectrophotometry or fluorometry. The unknown samples are compared to the known samples for quantitation.

Two well-known techniques for absolute quantitation are:

- TaqMan® assay chemistry (RNase P)
- SYBR® Green assay

Absolute quantitation using the TaqMan assay chemistry is a highly accurate technique for quantifying DNA. The TaqMan® DNA Template Reagents (PN 401970) and the TaqMan RNase P Detection Reagents (PN 4316831) allow for convenient means to quantify gDNA. The kit includes pre-diluted and validated standards at five concentrations per kit: 0.6 ng/µL, 1.2 ng/µL, 3.0 ng/µL, 6.0 ng/µL, 12.0 ng/µL. Dilute or aliquot to the appropriate range for your samples.

SYBR Green is a dye which binds only to double-stranded DNA (dsDNA). Quantitation with SYBR Green assay chemistry is less specific than TaqMan assay chemistry because the dye binds to any dsDNA whereas TaqMan reagent chemistry targets a specific DNA sequence. The SYBR Green method requires melt curve analysis to verify the specificity of the assay.

For either technique, be sure to run the standard curve and unknown samples on the same plates in the SDS instrument.

Fluorometric Analysis

You can quantitate DNA by fluorometric analysis using various intercalating dyes. These are summarized in Table 5-6 on page 5-9.

Table 5-6 Dyes for fluorometric quantitation of DNA

Dye	Features of the Dye
Hoechst dye #33258	• More sensitive than spectrophotometric measurements due to low affinity for RNA. • Base composition of the DNA can affect readings because the dye binds preferentially to AT-rich DNA (Gallagher 1994).
Ethidium bromide	• Not base composition sensitive • Binds to RNA • Capable of detecting nanogram levels of DNA • Ideal for relatively pure DNA with a high GC content (Gallagher 1994)
PicoGreen® dye	• Can quantitate as little as 25 pg/ml up to 1000 ng/mL of dsDNA

Assay Problems

These problems have to do with preparing the assay. They include:

- "Reagents Mishandled or Expired" on page 5-9
- "Using a Master Mix Without ROX™ Dye" on page 5-10
- "DNA or Assay Reagent Not Added to the Reaction Well" on page 5-10
- "Insufficient DNA Added to the Reaction Well" on page 5-11
- "Evaporation from the Reaction Well" on page 5-11
- "Pipetting Errors" on page 5-11
- "Inefficient Mixing and/or Insufficient Centrifugation" on page 5-12
- "Assay Has High Background Fluorescence" on page 5-12
- "More Than One Sample in the Well" on page 5-13

Reagents Mishandled or Expired

The use of mishandled or expired reagents may result in:

- Some or all samples clustering with the NTCs
- Trailing clusters
- Weak overall reaction (weak signals)

What to Do Perform the assay again with newly prepared reagents. Follow the guidelines below for reagent storage and handling.

Assay Considerations

- Store TaqMan Drug Metabolism Genotyping Assays at –15 to –25 °C when they are not in use.
- Minimize freeze-thaw cycles to no more than ten cycles. Too many freeze thaw cycles can cause cleavage of the dye from the probe.
- Limit the assay exposure to light. The fluorescent dyes are susceptible to photo-bleaching. Photo-bleaching can result in a lower overall signal for the reaction.

TaqMan® Universal PCR Master Mix Considerations

- Store TaqMan® Universal PCR Master Mix at 2-8 °C.
- Prior to use, make sure the Master Mix is thoroughly mixed.

Using a Master Mix Without ROX™ Dye

The use of a PCR Master Mix that does not contain ROX™ dye (or a similar passive reference) can cause:

- Trailing clusters
- Some or all data is undetermined (There is an "X" instead of a called allele in the AD plot.)
- Diffuse clusters

What to Do Use Applied Biosystems TaqMan® Universal PCR Master Mix which includes ROX dye.

Discussion ROX dye is a passive reference dye that improves the precision of the results by compensating for small fluorescent fluctuations, such as bubbles and small well-to-well variations.

On the 7900HT Fast Real-Time PCR System, the Sequence Detection Software will not call the alleles when ROX dye (or another passive reference) is not present.

See Also "ROX™ Dye Not Designated as Reference" on page 7-10

DNA or Assay Reagent Not Added to the Reaction Well

When gDNA or one of the assay reagents is not added to the reaction well, no PCR amplification takes place and the sample clusters with the NTCs.

What to Do Perform the assay again, making sure to:

- Follow the *TaqMan® Drug Metabolism Genotyping Assays Protocol* exactly
- Pipette carefully
- Mix thoroughly

Insufficient DNA Added to the Reaction Well

When insufficient gDNA is added to the reaction well, no PCR amplification takes place and the sample clusters with the NTCs.

What to Do

Perform the assay again, making sure to:

- Quantitate your DNA accurately (see "DNA Preparation" on page 2-2)
- Follow the *Taq®Man Drug Metabolism Genotyping Assays Protocol* as recommended, adding between 3 to 20 ng of purified genomic DNA
- Pipette carefully
- Mix thoroughly

Evaporation from the Reaction Well

Evaporation of your reaction can occur if the reaction plates are not properly sealed, leading to:

- Outliers (mild/moderate evaporation)
- Trailing clusters (moderate evaporation)
- Samples clustering at the NTC (extreme evaporation)

What to Do

1. Check the location of the wells for the problem calls. Evaporation can most often occur around the edges of the plate.
2. Check the seals of the optical adhesive cover for leaks.
3. If there are leaks, perform the assay again.

   Use an adhesive seal applicator (PN 4333183) to thoroughly seal the cover. Make sure to run the applicator over the edges of the seal.

Discussion

Evaporation can occur if your plate is not properly sealed. As evaporation occurs, the water in the reaction decreases, causing the signals from the reporter and ROX™ dyes to increase due to increased concentration of the dyes. The degree of evaporation influences the assay results:

- Mild – If the PCR reaction is not affected, the ROX dye can compensate for the increased signals and the assay will work correctly.
- Mild to moderate – You may see outlier samples. Depending on the number of wells affected, the plot may show only a few outliers or it may show a trailing cluster.
- Extreme evaporation occurring early in the reaction – The PCR reaction fails and the samples cluster with the NTC.

Pipetting Errors

Pipetting errors can cause inconsistent delivery of reagents or sample to the wells, which can cause:

- Trailing clusters
- Some (or all) samples clustering with the NTCs

- Cloudy or diffuse clusters
- A sample (or samples) that does not cluster with a specific allele (i.e. is an outlier)

What to Do

- Improve pipetting precision, as follows:
  - Calibrate and clean the pipettors regularly.
  - Pipette larger volumes (no less than 5 µL) for greater accuracy and precision.
  - Reduce the number of pipetting steps whenever possible.
  - Increase the consistency of the pipetting method (such as using robotic pipetting).
  - Consult the manufacturer about the correct method of dispensing liquid volumes accurately from the pipettor. For example, some pipettors are designed to deliver the designated volume at the first plunger stop, so "blowing out" the residual volume may cause error.
- Validate your pipetting process by preparing a replicate plate (same assay and sample over a plate) to be sure results are reproducible.

Inefficient Mixing and/or Insufficient Centrifugation

Insufficient mixing can cause trailing clusters in the AD plot.

What to Do Rerun the assay, mixing the samples well (by pipetting up and down a few times) and performing the centrifugation steps as described in the protocol. Centrifuging the samples ensures that the contents of the sample well are pooled at the bottom of the well, allowing for the most efficient PCR reaction and the most accurate endpoint read.

Assay Has High Background Fluorescence

Some assays have higher levels of background fluorescence than others. Results of high levels of background fluorescence:

- The position of the NTCs moves away from the origin of the allelic discrimination plot

  or
- The position of the homozygous cluster moves towards the heterozygous clusters.

What to Do Measure the Rn-NTC values for each cluster.

If the clusters are well separated from each other, the Sequence Detection Software can autocall the clusters. You can also manually call the clusters.

More Than One Sample in the Well

Sometimes samples are inadvertently mixed together due to poor lab technique, resulting in an outlier.

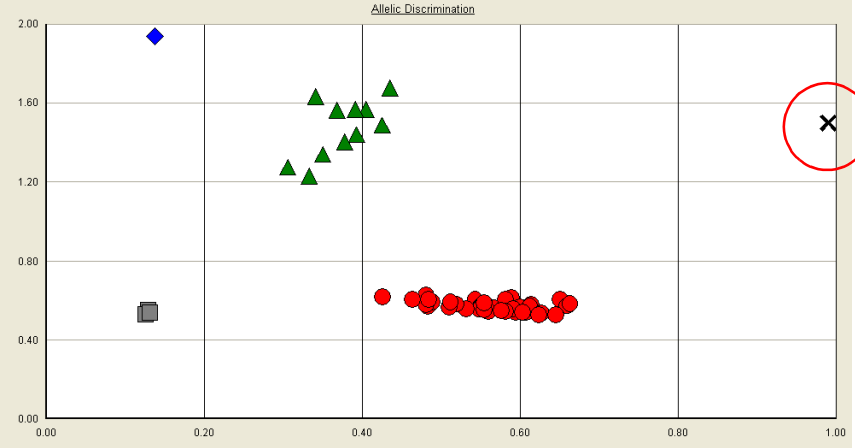


Figure 5-5 Allelic discrimination plot with different samples in the same well. Outlier sample circled in red.

What to Do

Perform the assay again, making sure you do not combine two samples.

6

Instrument Troubleshooting

To eliminate poor thermal cycler performance, ensure that you maintain instruments according to the recommendations presented in this chapter.

This chapter covers:

Introduction 6-2
Routine Thermal Cycler Maintenance 6-2
Instrument Calibration. 6-3

Introduction

For best results, your instrument needs to be up-to-date with calibration and maintenance.

Poor thermal cycler performance can result in:

- Trailing clusters
- Some or all samples cluster with the NTCs
- Cloudy or diffuse clusters
- High signal for the NTCs in one or more of the sample wells

Topics in this chapter will help you eliminate these problems.

Routine Thermal Cycler Maintenance

To ensure optimal performance of your thermal cycler or Sequence Detection System, it is strongly recommended that you perform routine maintenance. Maintenance schedules vary by instrument; see Table 6-1, below

Table 6-1 Summary of thermal cycler maintenance by instrument

Instrument	Details	Maintenance Interval
GeneAmp®PCR Systems 9700	• Clean the sample wells and the heated cover. • Run the Calibration Temperature Verification test • Run the Temperature Nonuniformity Test	Every 3 to 6 months
9800 Fast PCR System	• Replace fuses • Run the Calibration Temperature Verification test • Run the Temperature Nonuniformity Test	As needed
7900HT Fast Real-Time PCR System 7900HT Real-Time PCR System	Clean the sample block	As needed
7300/7500/7500 Fast Real-Time PCR Systems	• Clean the sample wells • Replace the halogen bulb • Replace fuses	As needed

Clean the Sample Block

CAUTION Before using a cleaning or decontamination method other than those recommended by the manufacturer, verify with the manufacturer that the proposed method will not damage the equipment.

Decontamination of the sample block is generally performed to resolve problematic background calibrations, where one or more wells consistently exhibit abnormally high signals, indicating the presence of a fluorescent contaminant.

Thermal Cycler Diagnostics

There are two tests you can perform on your thermal cycler:

- **Calibration Verification Test** – determines if the instrument is properly calibrated.
- **Temperature Nonuniformity Test** – determines how uniformly the block heats.

If either test fails, call Technical Support or open a service call.

You need the appropriate temperature verification kit to perform the tests.

For More Information

See "Thermal Cycler Maintenance and Calibration Documentation" on page vi.

Instrument Calibration

To ensure optimal performance of your thermal cycler or Sequence Detection System, it is strongly recommended that you regularly calibrate your thermal cycler. Calibration varies by instrument; see Table 6-2 on page 6-4

For More Information

See "Thermal Cycler Maintenance and Calibration Documentation" on page vi.

Types of Calibration

ROI Calibration

The ROI calibration allows the Sequence Detection Software to map the position of the wells on the sample block so that, during instrument operation, the software can associate increases in the fluorescence with specific wells of the reaction plates.

Background Calibration

The background calibration measures the ambient fluorescence that is generated from background electrical signals, samples blocks, water inside consumables, and from the consumables themselves. This calibration enables the Sequence Detection Software to eliminate background signal from the fluorescent samples, thus increasing the instrument's precision.

Pure Dye Spectra Calibration

The pure dye spectra calibration enables the instrument to distinguish the fluorescent dyes being used in the system. The Sequence Detection Software uses the spectral data from a set of pure dye standards to process the raw spectral data it receives after each run.

Instrument Verification Run

The test verifies that the instrument can generate a standard curve and its ability to calculate the quantities of two unknowns. This test requires an RNase P Verification Plate that contains pre-loaded reagents that create a standard curve with known copy numbers and two unknowns (also with known copy numbers).

Calibration Summary

Table 6-2 Summary of calibration by instrument

Calibration Type	Instrument	Interval
ROI	7300/7500/7500 Fast Real-time PCR Systems	• Every 6 months *or* • As needed to verify the instrument's performance
Background	7900HT Fast System 7900HT Real-Time PCR System 7300/7500/7500 Fast Real-time PCR Systems	• Every 6 months *or* • Before performing a pure dye calibration *or* • When installing an uncalibrated block
Pure dye spectra	7900HT Fast System 7900HT Real-Time PCR System 7300/7500/7500 Fast Real-time PCR Systems	• Every 6 months *or* • Before performing an instrument calibration run *or* • When installing an uncalibrated block
Instrument verification	7900HT Fast System 7900HT Real-Time PCR System 7300/7500/7500 Fast Real-time PCR Systems	• Every 6 months *or* • As needed to verify the instrument's performance

7

Troubleshooting Software Problems

Three conditions in allelic discrimination plots point to problems that occurred during software analysis. Most of these problems can be eliminated during software setup. This chapter provides troubleshooting suggestions for software setup.

This chapter covers:

Introduction 7-2
Empty Allelic Discrimination Plots 7-2
No Alleles Called in the AD Plot 7-4
Homozygous Allele Frequencies Reversed 7-15
Too Many Alleles Called in the AD Plot 7-21

Introduction

This chapter highlights common Sequence Detection Software issues that can lead to problems with TaqMan® Drug Metabolism Genotyping Assays. For complete instructions for setting up, running and analyzing a plate, please refer the user manual for your software.

The software analysis of your data is an important component of your project. Incorrect analysis of your data can undermine a successful assay by causing undetermined, ambiguous or incorrect genotypes. AD plot problems due to software fall into four broad categories, each of which is further discussed below.

- Empty allelic discrimination plots – page 7-2
- No alleles are called in the AD plot – page 7-4
- The homozygous allele frequencies are the reverse of expected – page 7-15
- Too many alleles are called in the AD plot – page 7-21

The Sequence Detection Software is different for each instrument you can use to read your plate. Be sure to follow the steps for your instrument in the What To Do sections below. If there are no instrument-specific instructions, the instructions are applicable for both versions of the software.

IMPORTANT! The instructions below are for specific versions of the Sequence Detection Software:

- For 7900 SDS, version 2.3
- For 7500 Fast System SDS, version 1.3

For More Information

Refer to the User Guide supplied with your software for additional information. See "SDS Documentation," on page vi for titles and part numbers.

Empty Allelic Discrimination Plots

Incorrectly creating and/or selecting the detector and marker can result in an empty allelic discrimination plot even when the assay chemistry is successful.

When the allelic discrimination plot is empty, causes include:

- No marker assigned to the well (7-2)
- A well (or wells) omitted during data collection or analysis (page 7-3)
- The reporter dye assigned incorrectly (page 7-15)

No Marker Assigned to the Well

A marker must be assigned to each well before SDS can analyze the plate and obtain results.

What to Do

If you have a post-read plate that appears to have no data:

1. Check to see if a marker is assigned.

2. If no marker is assigned, assign one and reanalyze the data.

   See the instructions appropriate for your software:

   - "Detectors, Markers and Tasks for the 7900HT Fast Real-Time PCR System" on page 2-6
   - "Detectors, Markers and Tasks for 7300/7500/7500 Fast Real-Time PCR Systems" on page 2-10

Omit Option Checked in Sequence Detection Software

If there is a red X in the plate document for a well, the Omit Well option may have been checked for this well.

The Omit Well option removes the selected well from the analysis.

What to Do

If you checked Omit Well:

- *Before* the run, uncheck **Omit Well** and re-run the plate read.
- *After* the run (during the analysis phase), follow the steps below.

For the 7900 SDS Software:

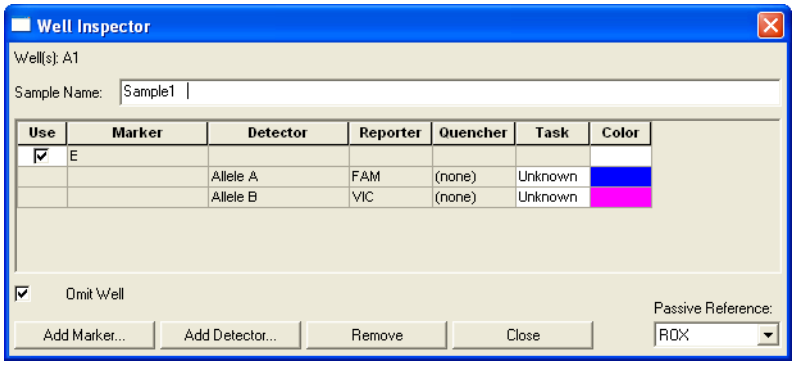
Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

1. In the plate document, click the cell corresponding to the well to select it.
2. Click the **Setup** tab to bring it to the front.
3. At the bottom of the Setup tab, uncheck **Omit Well**.
4. Click to reanalyze the plate.

For the 7500 Fast System SDS Software:

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

1. Click the **Setup** tab to bring it to the front.
2. In the plate document, double-click the well to open the Well Inspector dialog box.
3. Uncheck **Omit Well** then click **Close**.



4. Click to reanalyze the plate.

No Alleles Called in the AD Plot

To assist with cluster calling, the SDS Software can autocall the data. When autocalling fails, you will see "X" on the plot rather than the symbol for called alleles.

There are some instances where the software will not autocall the data:

- Autocalling option not selected (page 7-4)
- Autocalling after manual calling in the 7500 Fast System SDS Software (page 7-5)
- 2 cluster calling not selected (page 7-8)
- Single cluster (page 7-8)
- ROX™ dye not selected as passive reference (page 7-5)
- An outlier data point is too far off scale (page 7-12)
- NTCs are not assigned (page 7-14)

Autocall is Not Selected

The software will not autocall unless the "autocall" option is selected. If "autocall" is not selected, the allelic discrimination plot shows all the calls as X.

What to Do

For the 7900 SDS Software:

Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

1. Select **Analysis ▸ Analysis settings**.
2. Check **Auto Caller Enabled**.
3. Click **OK** and then to reanalyze the plate.

For the 7500 Fast System SDS Software:

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

1. Select **Analysis ▸ Analysis settings**.
2. Select **Automatic allele calling**.
3. Click **OK** and then to reanalyze the plate.

Autocalling After Manual Calling

For the 7900 Fast System SDS Software

The 7900 Fast System SDS Software is capable of autocalling after manual calling.

For the 7500 Fast SDS Software

If you do manual calling followed by autocalling, the autocalling will fail.

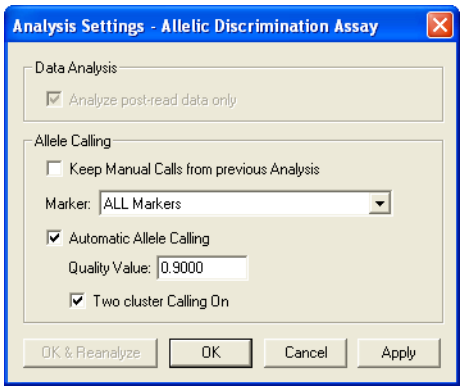
What to Do

For the 7500 Fast SDS Software

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

After manual calling:

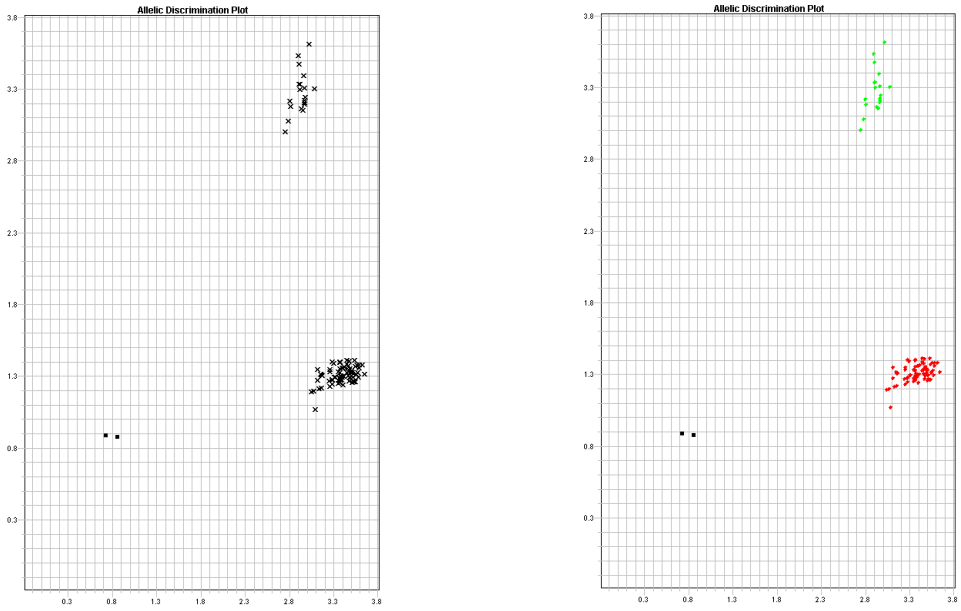
1. Select **Analysis** ▸ **Analysis settings**.



2. Uncheck **Keep Manual Calls from previous Analysis**.
3. If needed, check **Automatic Allele Calling**.
4. Click **OK** and then click to reanalyze the plate.

Two Cluster Calling Not Selected

The “2 cluster calling” option in the SDS Software must be selected if the software is to successfully autocall plates when only two clusters are detected. (Detection of only two clusters can happen if the MAF is low and/or you ran too few samples to detect all three genotypes.) If this is the case, you must select the two cluster calling option and reanalyze the plate.



Without 2 cluster calling enabled

With 2 cluster calling enabled

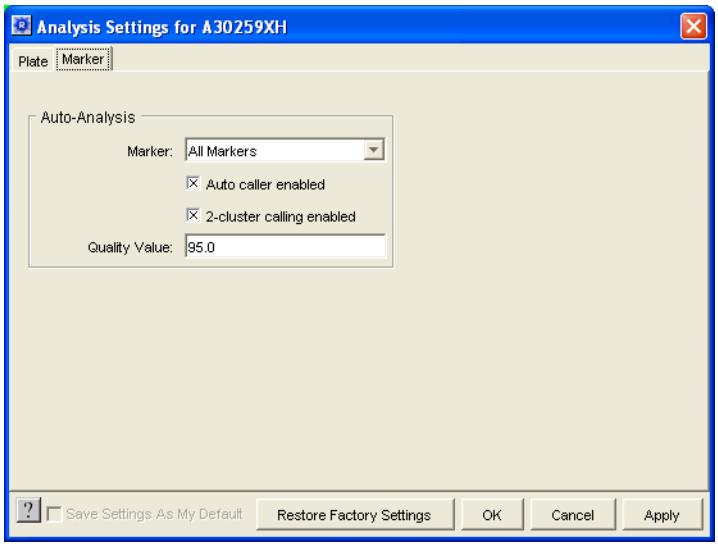
Figure 7-1 Allelic discrimination plots before and after selecting the “2 cluster calling” option

What to Do

For the 7900 Fast System SDS Software

Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

1. Select **Analysis ▸ Analysis settings**.

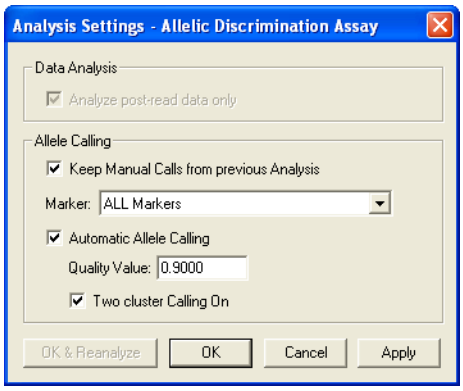


2. Select **2 cluster calling enabled**.
3. Click **OK** and then click to reanalyze the plate.

For the 7500 Fast SDS Software

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

1. Select **Analysis ▸ Analysis settings**.



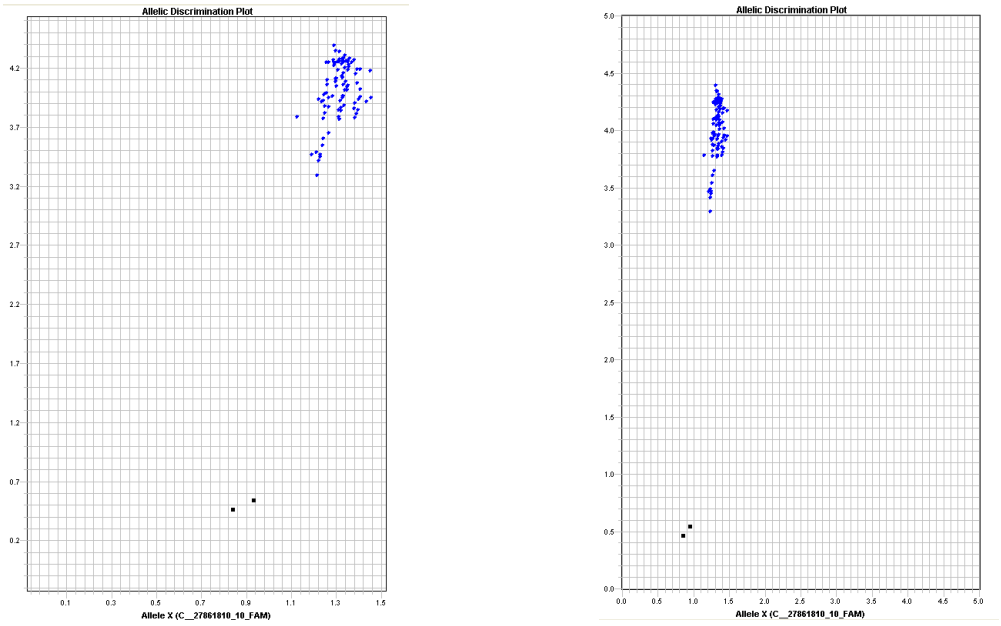
2. Select **Two cluster Calling On**.
3. Click **OK** and then click to reanalyze the plate.

See Also "Low Allele Frequency" on page 4-2.

Single Cluster Assay

The SDS Software cannot autocall single cluster assays. For many assay/sample combinations, a single cluster assay is the correct result. For example, a single cluster assay can be correct for a SNP with a very low minor allele frequency (see "Low Allele Frequency" on page 4-2). Many of the TaqMan® Drug Metabolism Genotyping Assays have a very low minor allele frequency, so you may see single clusters in your experiments.

If you believe the single cluster is correct, manually call the alleles. You may need to rescale the plot before you can call the alleles accurately.



Single cluster, before rescaling

Single cluster, after rescaling

Figure 7-2 Allelic discrimination plots before and after rescaling

What to Do

1. If needed, click the **Results** tab to bring it to the front.
2. Double-click the plot. In the resulting dialog box, uncheck **Autoscale**. Enter numbers for the scaling, then click **Apply**.

   The software rescales the plot so you can see to which axis of the plot the data is nearer.

   If the plot is rescaled sufficiently, click **OK**. If not, rescale the plot again.
3. Click the **Arrow** or **Lariat** tool.

4. Depending on the tool you selected, choose the data points you want to autocall:
   - For the Arrow – Click and drag to draw a box.
   - For the Lariat – Click and drag to draw a free-form circle.

5. In the **Call** pull-down menu, select the allele call that you want to assign to the selected data points.

Assign the allele based on the nearness of the data point(s) to the reporter dye axis of the AD plot.

ROX™ Dye Not Designated as Reference

When ROX™ dye is not designated as a reference:

- For the 7900 SDS Software, the software will not autocall the alleles.
- For the 7500 Fast System SDS Software, you will see trailing clusters in the allelic discrimination plots, as shown in Figure 7-3 on page 7-10.

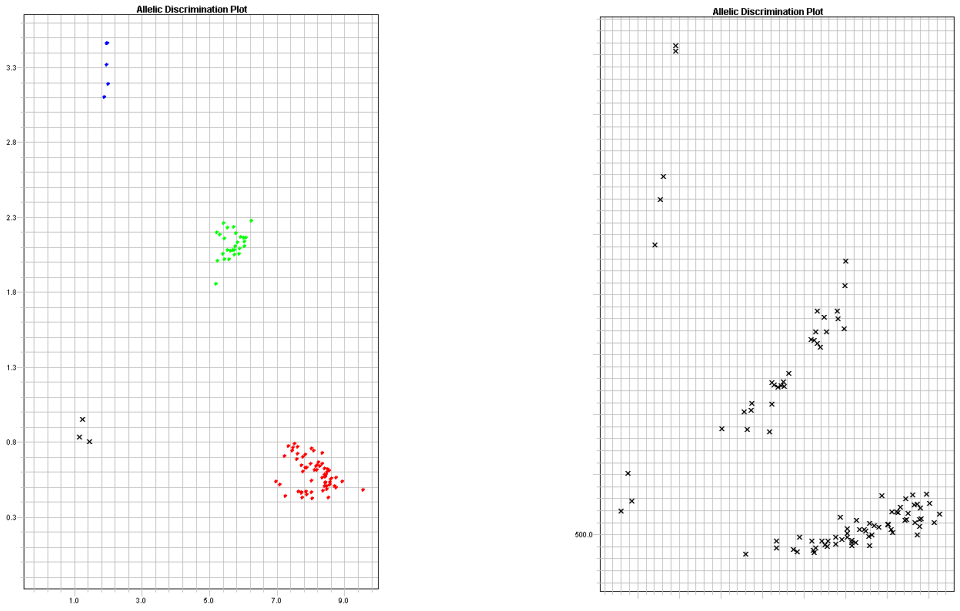


Figure 7-3 Data analyzed with and without ROX™ dye as passive reference

What to Do

For the 7900 SDS Software:

Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

1. Click the well of interest to select it.
2. Click the **Setup** tab.
3. At the bottom of the tab, select ROX in the **Passive reference** list.
4. Click to reanalyze the plate.

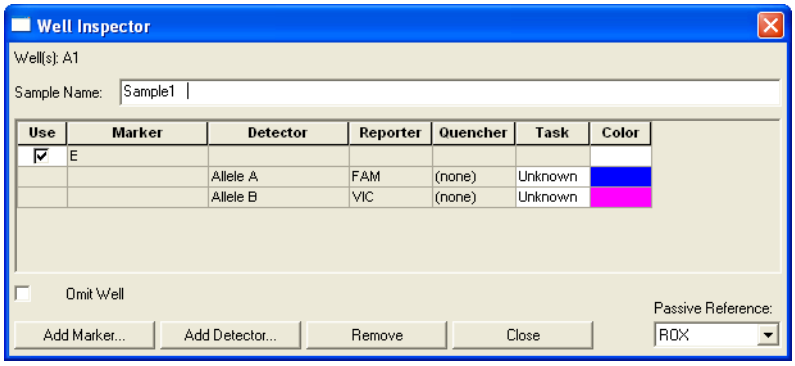
For the 7500 Fast System SDS Software:

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

1. Click the **Setup** tab to bring it to the front.

2. In the plate document, double-click the well.

   The Well Inspector dialog box appears.



3. For **Passive Reference**, select ROX, then click **Close**.
4. Click to reanalyze the plate.

Discussion

ROX™ dye is a passive reference that improves the precision of the results by compensating for small fluorescent fluctuations, such as bubbles and small well to well variations, that occur in the plate.

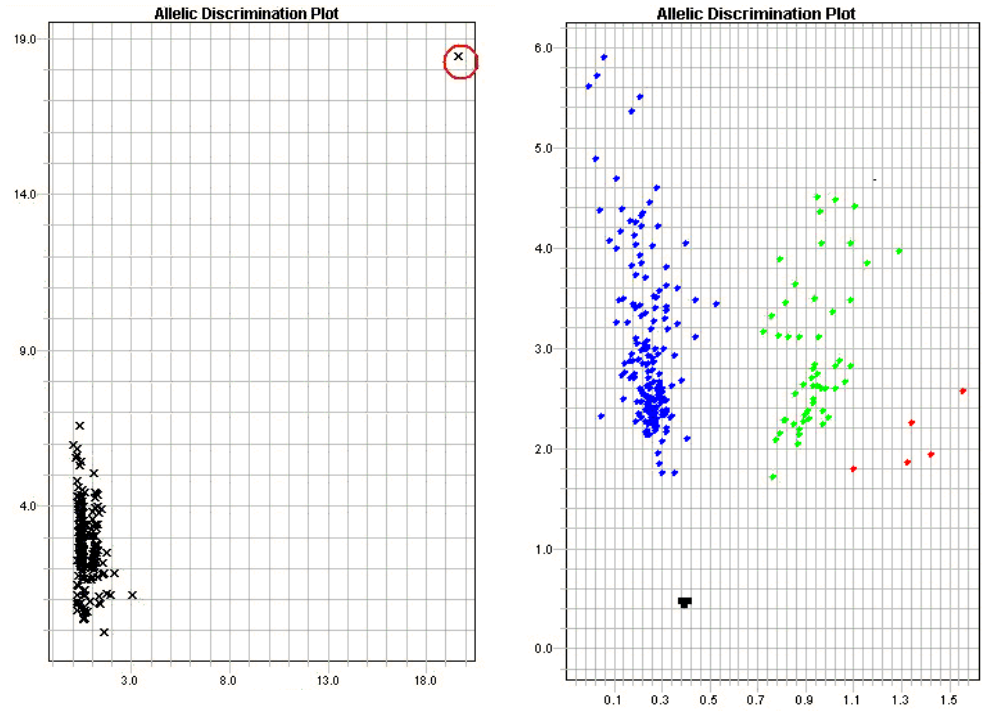
- The 7900 SDS Software requires that a reference dye is selected in order to autocall.
- The 7500 Fast System SDS Software does not require the presence of ROX dye in order to autocall, but its presence will improve the results.

See Also

"Using a Master Mix Without ROX™ Dye" on page 5-10.

Outlier Too Far Off Scale

If you have an assay that shows clustering around the NTCs, you may want to look for data from an outlier sample. In some cases, the software scales to include the outlier giving the other samples the appearance of clustering around the NTC. If you remove the outlier from the analysis, the program rescales the data and the analysis can proceed.



Outlier circled in red

Data reanalyzed with outlier omitted

Figure 7-4 Data analyzed with and without outlier included. (This example is not a TaqMan® Drug Metabolism Genotyping Assay but is included for here illustration.)

What To Do

Remove the outlier(s) and reanalyze. The program will adjust the scaling.

For the 7900 SDS Software:

Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

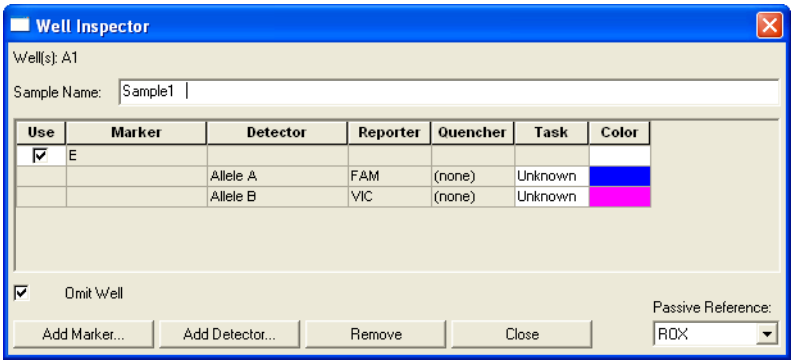
1. If needed, click the **Results** tab to bring it to the front.
2. In the allelic discrimination plot, click and drag the mouse to select the outlier.
3. Click the **Setup** tab to bring it to the front.
4. Check **Omit Well(s)**.
5. If there are additional outliers, repeat steps 2 through 4.
6. Click to reanalyze the plate.

For the 7500 Fast System SDS Software

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

1. If needed, click the **Results** tab to bring it to the front.
2. In the allelic discrimination plot, click and drag the mouse to select the outlier.
3. Click the **Setup** tab to bring it to the front.
4. In the plate document, double-click the selected well.

   The Well Inspector dialog box appears.



5. Click **Omit Well** and then **Close**.
6. If there are additional outliers, repeat steps 2 through 5.
7. Click to reanalyze the plate.

See Also "Low Allele Frequency" on page 4-2.

NTCs Not Assigned

If the wells containing the NTCs are not assigned with the NTC task in the software, the software may not call the alleles.

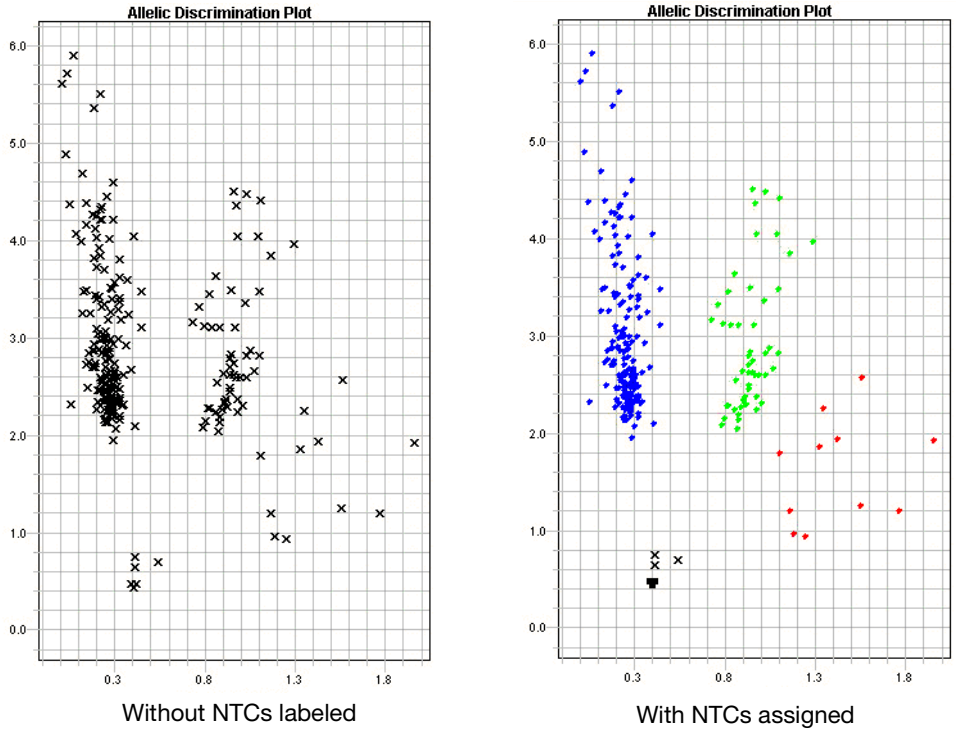


Figure 7-5 Data analyzed with and without the NTCs assigned. (Note that this example is not a TaqMan® Drug Metabolism Genotyping Assay, but is included here for illustration.)

What to Do

Assign the NTC task to the NTC wells in the plate and reanalyze the data. See the instructions appropriate for your software:

- "Detectors, Markers and Tasks for the 7900HT Fast Real-Time PCR System" on page 2-6
- "Detectors, Markers and Tasks for 7300/7500/7500 Fast Real-Time PCR Systems" on page 2-10

Discussion

In some instances where the data is diffuse and software does not autocall, labeling the NTC wells with the NTC task provides a point of reference for the software, improving clustering and autocalling.

Note: NTCs are not required for autocalling.

Homozygous Allele Frequencies Reversed

Your observed major and minor allele frequencies for homozygotes are reversed from those predicted by the Hardy-Weinberg Equilibrium equation. For example, for a SNP with a MAF of 5% (0.05), the predicted frequencies are 0.0025 q:q, 0.095 q:p, and 0.9025 p:p. If the allele frequencies are reversed, you see 0.9025 q:q, 0.095 q:p, and 0.0025 p:p.

Reporter Dye Assigned Incorrectly to Allele in Detector

In the software, you set up two detectors and one marker in order for the alleles to be called. The detector defines which dye is assigned to the allele. If you inadvertently assigned the dyes to the wrong alleles when you created the detectors, the observed frequencies will be the reverse of those predicted from the Hardy-Weinberg equation.

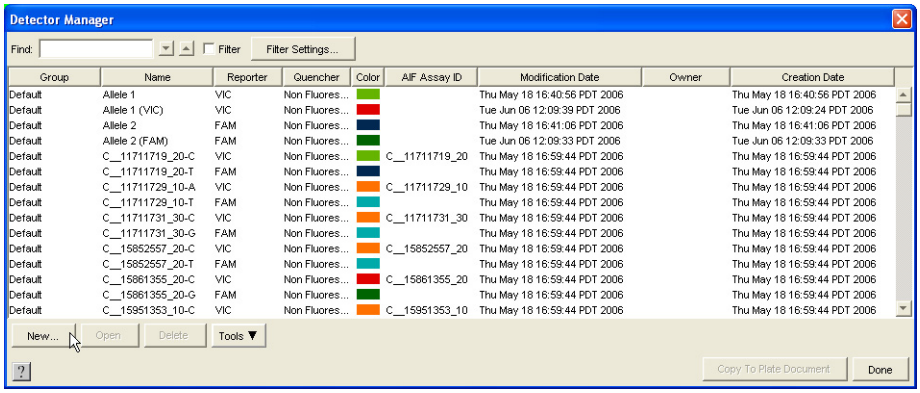
Note: The AIF included with your order contains the correct allele-dye assignments.

What to Do

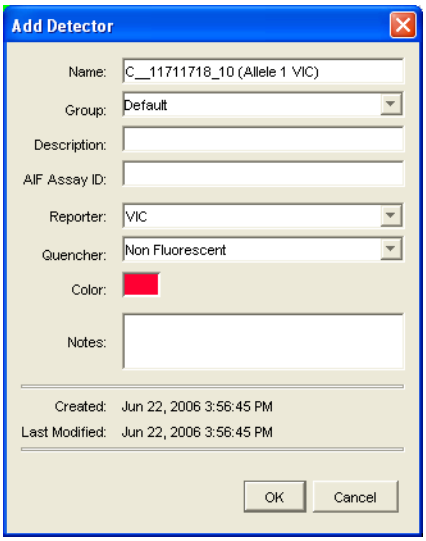
For the 7900 SDS Software:

Note: The instructions below are for Sequence Detection Software v.2.3. For other versions, see your user guide.

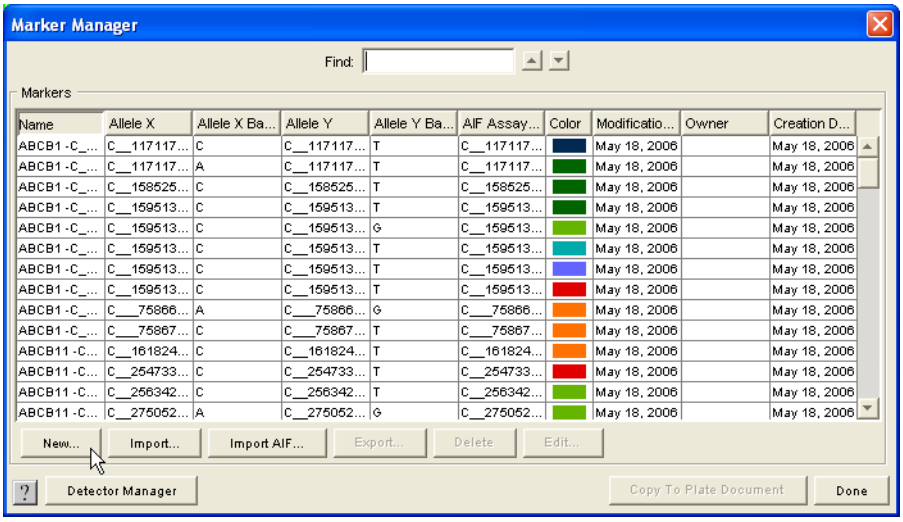
1. In the SDS Software, select **Tools** ▸ **Detector Manager** to open the Detector Manager dialog box.


Detector Manager
Find:
Filter
Filter Settings...
Group
Name
Reporter
Quencher
Color
AIF Assay ID
Modification Date
Owner
Creation Date
Default Allele 1 VIC Non Fluores... Thu May 18 16:40:56 PDT 2006 Thu May 18 16:40:56 PDT 2006
Default Allele 1 (VIC) VIC Non Fluores... Tue Jun 06 12:09:39 PDT 2006 Tue Jun 06 12:09:24 PDT 2006
Default Allele 2 FAM Non Fluores... Thu May 18 16:41:06 PDT 2006 Thu May 18 16:41:06 PDT 2006
Default Allele 2 (FAM) FAM Non Fluores... Tue Jun 06 12:09:33 PDT 2006 Tue Jun 06 12:09:33 PDT 2006
Default C__11711719_20-C VIC Non Fluores... C__11711719_20 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711719_20-T FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711729_10-A VIC Non Fluores... C__11711729_10 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711729_10-T FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711731_30-C VIC Non Fluores... C__11711731_30 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711731_30-G FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15852557_20-C VIC Non Fluores... C__15852557_20 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15852557_20-T FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15861355_20-C VIC Non Fluores... C__15861355_20 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15861355_20-G FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15951353_10-C VIC Non Fluores... C__15951353_10 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
New...
Open
Delete
Tools
Copy To Plate Document
Done

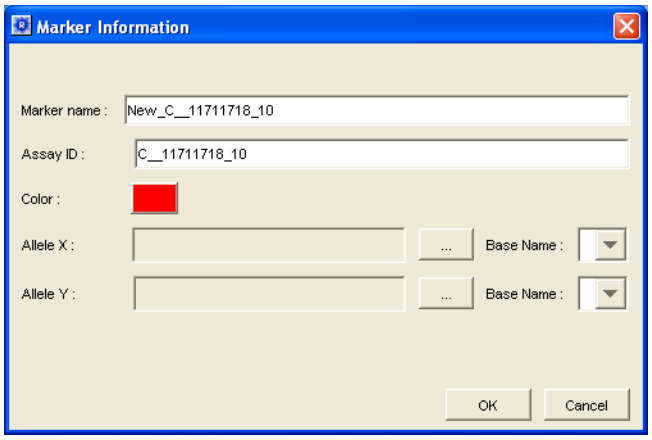
2. In the Detector Manager dialog box, click **New** to create a new marker.



3. Enter the information for the allele 1 detector, then click **OK**.
4. Repeat steps 2 and 3 for the allele 2 detector.
5. Click **Done** to close the detector manager
6. Select **Tools** ▸ **Marker Manager** to open the Marker Manager dialog box.

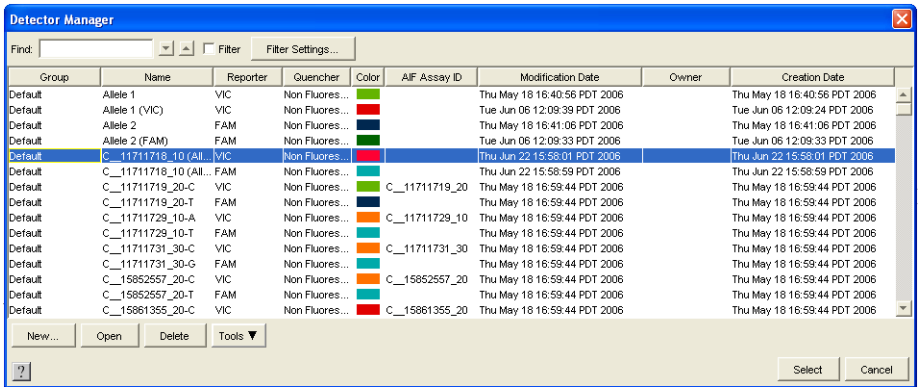


7. In the Marker Manager, click **New** to open the Marker Information dialog box.



a. Enter the marker name and, optionally, the assay name.

b. To choose the allele for the X axis of the allelic discrimination plot, open the Detector Manager dialog box by clicking ... next to the Allele X text field.

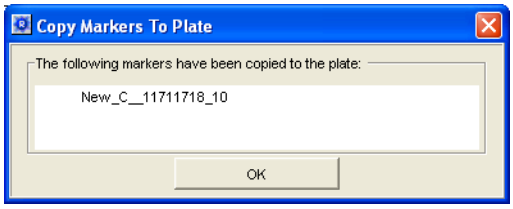

Detector Manager
Find:
Filter
Filter Settings...
Group
Name
Reporter
Quencher
Color
AIF Assay ID
Modification Date
Owner
Creation Date
Default Allele 1 VIC Non Fluores... Thu May 18 16:40:56 PDT 2006 Thu May 18 16:40:56 PDT 2006
Default Allele 1 (VIC) VIC Non Fluores... Tue Jun 06 12:09:39 PDT 2006 Tue Jun 06 12:09:24 PDT 2006
Default Allele 2 FAM Non Fluores... Thu May 18 16:41:06 PDT 2006 Thu May 18 16:41:06 PDT 2006
Default Allele 2 (FAM) FAM Non Fluores... Tue Jun 06 12:09:33 PDT 2006 Tue Jun 06 12:09:33 PDT 2006
Default C__11711718_10 (All... VIC Non Fluores... Thu Jun 22 15:58:01 PDT 2006 Thu Jun 22 15:58:01 PDT 2006
Default C__11711718_10 (All... FAM Non Fluores... Thu Jun 22 15:58:59 PDT 2006 Thu Jun 22 15:58:59 PDT 2006
Default C__11711719_20-C VIC Non Fluores... C__11711719_20 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711719_20-T FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711729_10-A VIC Non Fluores... C__11711729_10 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711729_10-T FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711731_30-C VIC Non Fluores... C__11711731_30 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__11711731_30-G FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15852557_20-C VIC Non Fluores... C__15852557_20 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15852557_20-T FAM Non Fluores... Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
Default C__15861355_20-C VIC Non Fluores... C__15861355_20 Thu May 18 16:59:44 PDT 2006 Thu May 18 16:59:44 PDT 2006
New...
Open
Delete
Tools
Select
Cancel

c. Click the allele 1 detector, click **Select**.

d. Repeat steps 7b and 7c to choose the allele 2 detector for the Y axis.

8. Copy the markers to the plate. In the Marker Manager, click the marker you just created to select it, then click **Copy to Plate**.

   The following message opens.

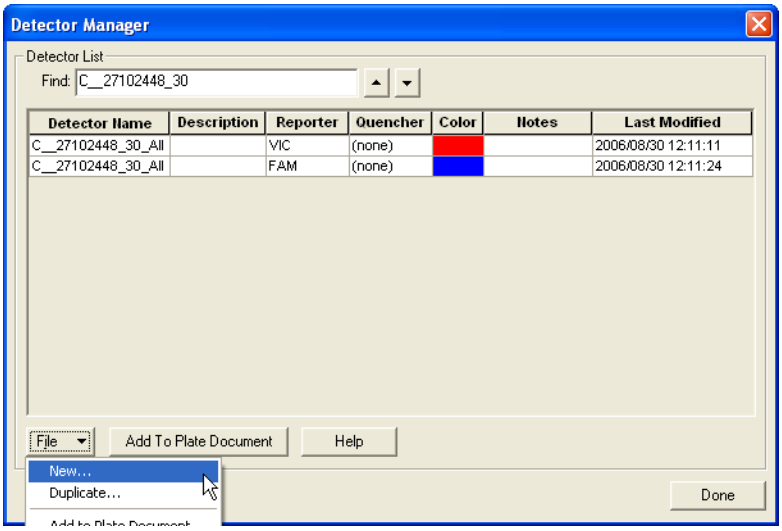


9. Click **OK**, then **Done**.
10. In the plate document, select the samples for which you want to replace the marker.
11. Uncheck the incorrect marker name and check the new marker name.
12. Click to reanalyze the plate.

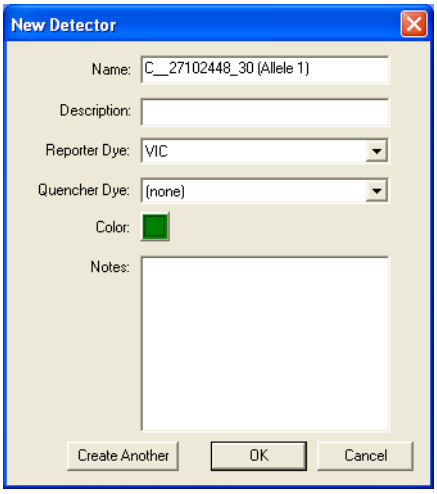
For the 7500 Fast System SDS Software:

Note: The instructions below are for Sequence Detection Software v.1.3. For other versions, see your user guide.

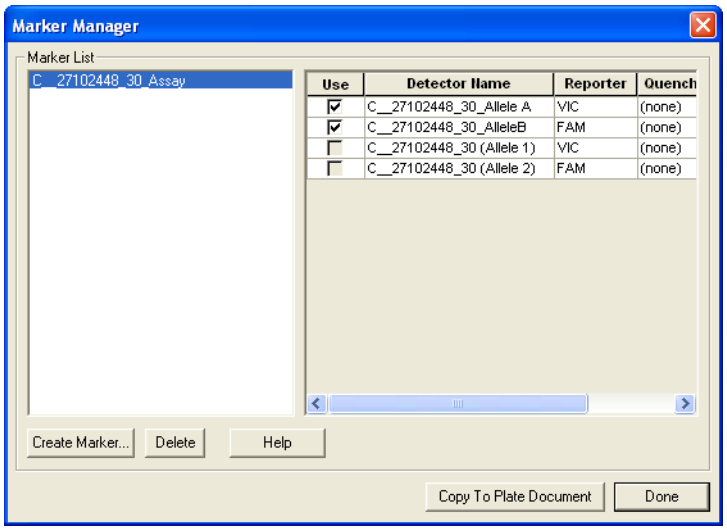
1. In the SDS Software, select **Tools** ▸ **Detector Manager** to open the Detector Manager dialog box.
2. Select **File** ▸ **New** to open the Detector Manager dialog box.



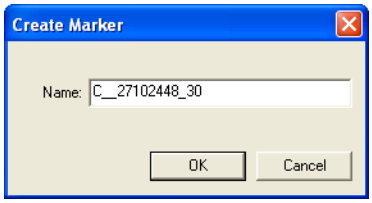
3. In the New Detector dialog box, enter information for the allele 1 detector.



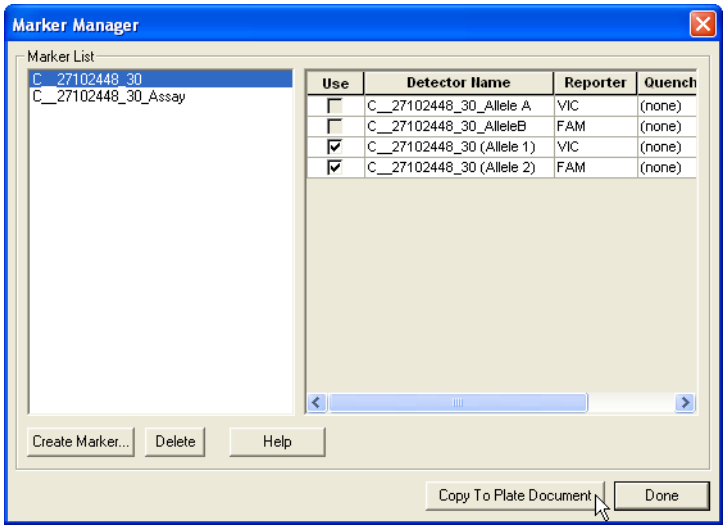
4. Click **Create Another** and enter the detector information for allele 2. When you are finished, click **OK**, and **Done** in the Detector Manager dialog box.
5. Select **Tools** ▸ **Marker Manager** to open the Marker Manager dialog box.



6. In the Marker Manager dialog box, click **Create Marker**.

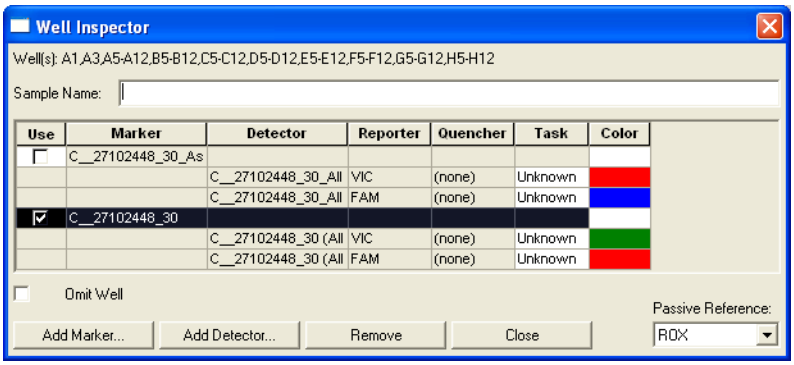


7. Enter the marker name, then click **OK**.
8. In the Marker Manager dialog box, click the name of the marker you just created. In the **Use** column, check the detectors that you created in steps 3 and 4.



9. Click **Copy To Plate Document**, then click **Done**.
10. In the plate document, click the **Setup** tab to bring it to the front.
11. Select the wells that you want to change, using the **Ctrl** and/or **Shift** keys to select wells individually or in groups.

12. While holding down the **Ctrl** and/or **Shift** keys, double-click on a well to open the Well Inspector dialog box. In the Well Inspector dialog box, assign the new marker to the selected wells.
    a. Click the **Use** check box next to the name of the old marker name to unselect it.
    b. Click the **Use** check box next to name of the new marker.
    c. Click **Close**.



13. Click to reanalyze the plate.

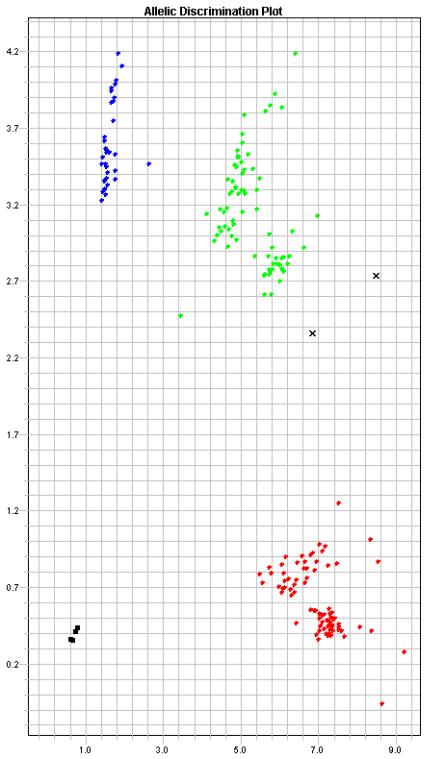
Discussion

For the 7900 SDS Software:

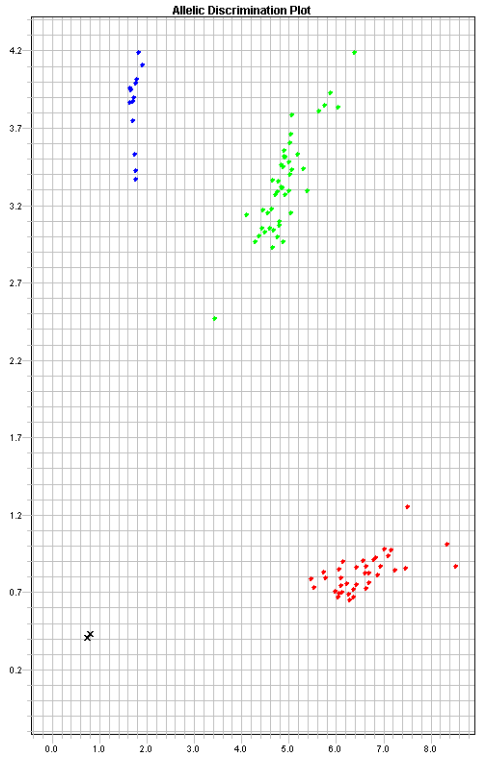
The SDS Software provides a wizard for creating your detectors and markers. If you use the Assay Information File button in the wizard, which reads the AIF supplied with your assay, the detectors and markers are automatically set up correctly.

Too Many Alleles Called in the AD Plot

In the software, you must set up one marker for each assay run on the plate. Running more than one assay per marker can result in more than three clusters in the allelic discrimination plot.



The same markers and detectors assigned to two assays.



The same markers and detectors assigned to one of the two assays.

Figure 7-6 Allelic discrimination plots showing two assays assigned to one detector and marker

What to Do

1. Create markers and detectors for *each* assay on the plate See the instructions appropriate for your software:
   - "Detectors, Markers and Tasks for the 7900HT Fast Real-Time PCR System" on page 2-6
   - "Detectors, Markers and Tasks for 7300/7500/7500 Fast Real-Time PCR Systems" on page 2-10
2. Assign each marker to the correct assays (see "Reporter Dye Assigned Incorrectly to Allele in Detector" on page 7-15).
3. Reanalyze your data.

Discussion

It is essential that each assay is assigned its own marker. Each assay has its own unique run characteristics. Running two assays with the same marker name may result in genotyping miscalls and the appearance of assay failure.

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